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Exosomes derived from menstrual blood-derived mesenchymal stem cells and treadmill training restore motor function following SCI by activating PI3K/Akt pathway

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Abstract

Background Spinal cord injury (SCI) remains a global challenge due to limited neural regeneration and functional recovery. Emerging therapies, such as mesenchymal stem cell-derived exosomes and exercise training, have shown promise, but their individual efficacy is insufficient. The synergistic effects of menstrual blood-derived mesenchymal stem cell-derived exosomes (MenSCs-Exo) and weight-supported treadmill training (WSTT) in SCI repair remain unclear. This study investigated their combined therapeutic potential and underlying mechanisms in SCI rats.

Methods A T10 spinal cord hemisection model was conducted in adult male Sprague-Dawley rats, which were randomized into five groups: Sham, SCI, Exo (200 μg MenSCs-Exo via tail vein injection every 48 h for 4 doses), TT (WSTT starting on day 3 post-SCI), and Exo + TT. Motor function was evaluated using the Basso, Beattie, and Bresnahan (BBB) scale and CatWalk XT® gait analysis. Histological assessments included hematoxylin and eosin (H&E) staining, Masson's trichrome staining, immunofluorescence for β-tubulin III (Tuj1) and myelin basic protein (MBP), and transmission electron microscopy (TEM). Western blot analyzed fibrosis-related proteins (COL1, COL3, α-SMA) and PI3K/AKT pathway activation (p-AKT, PI3K, β-catenin, LEF1).

Results Combined Exo + TT significantly improved motor function compared to monotherapies. BBB scores in the Exo + TT group were higher than SCI controls from day 7, with marked differences at 4 weeks ($P < 0.05$). CatWalk analysis revealed enhanced hindlimb coordination, reduced dragging, and improved paw print parameters in

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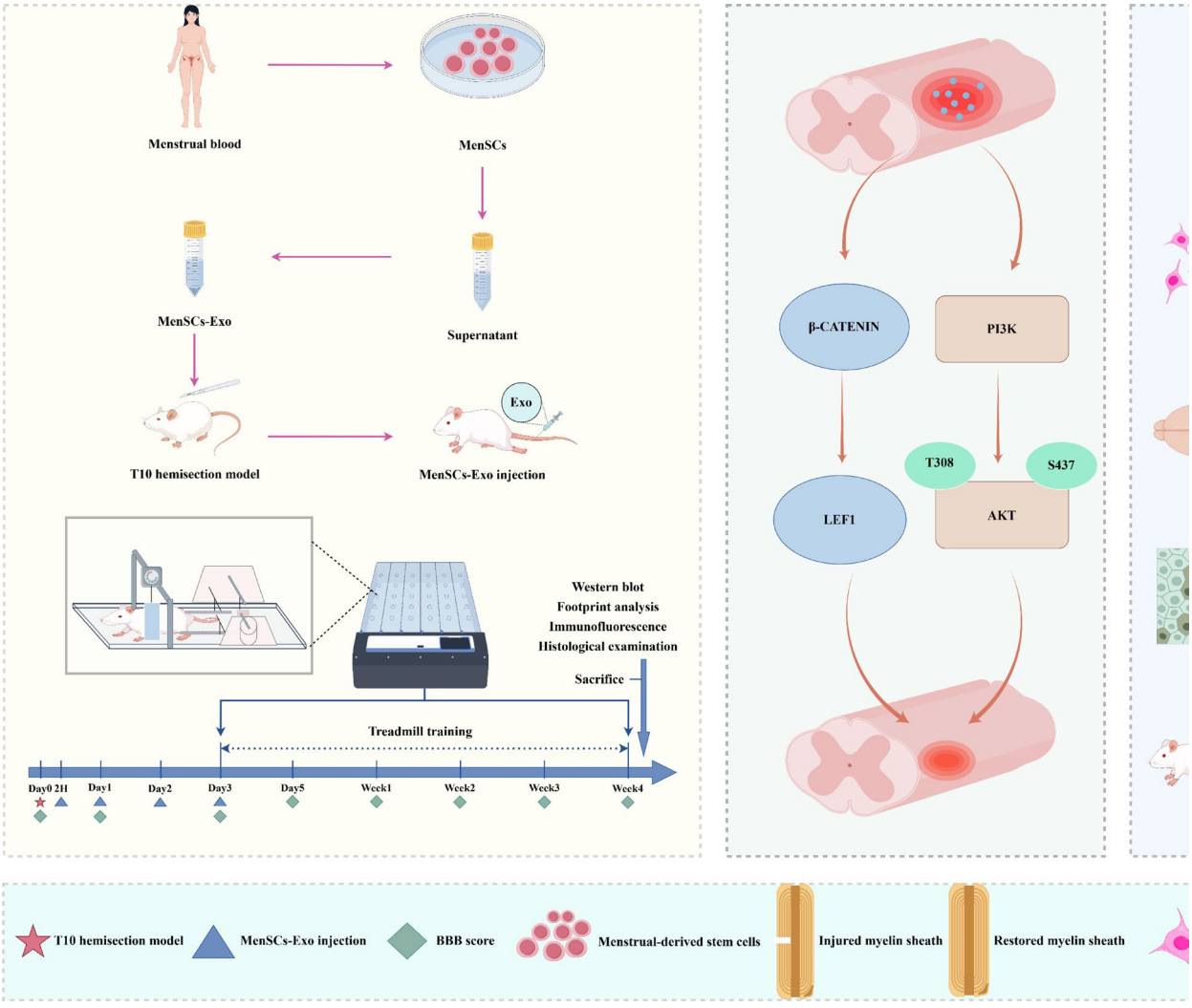
Exo + TT rats. Histologically, Exo + TT reduced spinal cord cavitation, inflammation, and fibrosis ($P < 0.01$ vs. SCI), while promoting axonal (Tuj1) and myelin (MBP) regeneration with ordered structure. TEM showed preserved myelin lamellae and reduced axonal degeneration. Western blot confirmed decreased COL1, COL3, and α-SMA expression, along with upregulated p-Akt, PI3K, β-catenin, and LEF1 in Exo + TT rats ($P < 0.01$).

Conclusion MenSCs-Exo combined with WSTT synergistically enhances motor recovery after SCI by promoting tissue repair, reducing fibrosis, and activating the PI3K/Akt pathway. This cell-free therapy paired with rehabilitation exercise offers a novel strategy for SCI treatment.

Keywords Spinal cord injury, Exosomes, Treadmill training, Menstrual blood-derived mesenchymal stem cells, PI3K/Akt signaling

Graphical abstract



Introduction

Spinal cord injury (SCI) is a severe, irreversible neurological disorder that impairs motor, sensory, and autonomic functions below the injury site, resulting in persistent organ dysfunction, chronic neurological deficits, and complications such as paralysis, pain, spasticity, and bowel/bladder dysfunction. These outcomes profoundly diminish patients' quality of life and impose considerable medical and economic burdens. Between 1990 and 2019, the global prevalence, incidence, and years lived with disability (YLD) due to SCI rose significantly, establishing SCI as a leading cause of premature mortality and long-term disability [1]. Moreover, individuals with SCI often experience depression and anxiety [2].

Current therapeutic approaches for neurological dysfunction following SCI include surgical decompression, pharmacotherapy, and rehabilitation; however, none of these approaches have been able to effectively enhance its functionality [3]. A diverse range of stem cells has been employed in the experimental treatment of SCI, encompassing bone marrow mesenchymal stem cells, umbilical mesenchymal stem cells, adipose-derived mesenchymal stem cells, neural stem cells, neural progenitor cells, embryonic stem cells, induced pluripotent stem cells, and extracellular vesicles. Each cellular subtype is tailored to address specific pathological characteristics of SCI, exerting therapeutic benefits through mechanisms such as cell replacement, trophic support, scaffold formation, and immunomodulatory regulation [3]. Although these strategies show promise, single interventions rarely restore impaired neuronal function due to limitations such as poor portability, adverse side effects, and modest clinical translation [4]. In recent years, combination therapies have demonstrated greater efficacy in promoting neural circuit restoration and functional recovery [4, 5]. Mesenchymal stem cells (MSCs), their exosomes, and exercise have gained traction in SCI treatment [6, 7]; however, the therapeutic and mechanistic benefits of MenSCs-derived exosomes (MenSCs-Exo) combined with exercise remain unclear.

Menstrual blood-derived mesenchymal stem cells (MenSCs), first isolated from the menstrual endometrium in 2007 [8], offer advantages over other MSCs, including abundant sources, non-invasive collection, robust differentiation capacity, high proliferation, genetic stability, and extensive secretion of neurotrophic factors, along with minimal ethical concerns for autologous transplantation. Previous studies revealed that MenSCs transplantation enhances tissue repair and neurological function in SCI rats [9, 10]. However, as with other stem cell therapies, MenSCs alone face limited homing, immunological rejection, and potential tumorigenesis, restricting the formation of functional connections [11]. Conversely, MenSCs-Exo exhibit low immunogenicity, negligible tumorigenicity, high safety, and the ability to cross the blood-brain and blood-spinal cord barriers, emerging as a valuable approach for SCI therapy.

Exercise is widely recognized as an effective rehabilitation strategy that improves motor function after SCI. Research suggests that exercise reduces blood-spinal cord barrier permeability, decreases tissue damage, and promotes angiogenesis, thereby enhancing functional outcomes compared with untreated SCI [12]. Furthermore, pairing exercise with stem cells or exosomes can yield synergistic benefits: treadmill training combined with adipose-derived MSC transplantation fosters angiogenesis and locomotor function in severe SCI [13], while exercise plus MSC-derived exosomes limits neuronal apoptosis and infarct size and supports synaptic and axonal remodeling in cerebral ischemia [14]. Rehabilitation programs such as locomotor training [15] and spatiotemporal epidural electrical stimulation have proven their clinical efficacy following incomplete SCI and/or complete paralysis [16]. Incorporation of rehabilitation is necessary for full function return [17].

In the central nervous system, axonal regeneration is regulated by the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR pathway, which is inhibited post-SCI, potentially hindering the protein synthesis required for axon repair [18]. The PI3K/Akt pathway serves a complex role: its activation in the subacute phase suppresses inflammation and apoptosis, whereas its inhibition in the chronic phase reduces glial scar formation [19]. Hence, enhancing PI3K/Akt activation early and restraining it later may represent an effective SCI treatment strategy.

This study aimed to determine whether MenSCs-Exo combined with treadmill training (TT) surpasses single treatments in improving SCI outcomes in rats and to clarify whether their synergistic effect is driven by PI3K/Akt activation. The study's conceptual framework and core design are visually summarized in Graphical Abstract.

Materials and methods

Cell culture and exosome isolation

MenSCs were isolated, cultured, and characterized using our established protocol [9]. Briefly, menstrual blood from healthy reproductive-aged female volunteers (day 2 of menstruation) was collected aseptically, followed by cell separation, culture, expansion, and passaging. MenSCs were identified via flow cytometry for stem cell surface markers and multi-differentiation potential via adipogenic, osteogenic, and chondrogenic induction assays. Cells at passages 6–7 were used for experiments. A stable GFP-expressing MenSCs line were established and the method has been described in detail in our previously published article [10]. Briefly, MenSCs were infected with lentiviruses carrying the GFP gene, followed by selection with puromycin. MenSCs stably expressing GFP were chosen for subsequent experiments. GFP-carrying exosomes were isolated from the supernatant of GFP-stable cell lines (MenSCs-GFP), identified, and reserved for use.

MenSCs were cultured to 80–90% confluence, and washed with PBS for three times, then replenished with exosome-free serum (EXO-FBS-250 A-1; System Biosciences, Mountain View, CA) containing DMEM/F12 medium. After 24 h, culture supernatants were collected into 50 mL tubes. Large debris and dead cells were removed by filtering through a 0.2 μm filter. Small-cell debris was removed by centrifugation at 10,000×g for 30 min, and exosomes were isolated by

ultracentrifugation at 100,000 g for 60 min to pellet exosomes. The total protein concentration was checked to quantitate the content of exosomes with a BCA kit (Beyotime Biotechnology, Shanghai, China). Exosomes were allocated into 1.5 mL EP tubes and stored at -80℃ for further use.

Exosome characterization

Exosomal markers (CD9, CD63, CD81, TSG101) were detected by Western blot. Morphology and size were observed via JEM-1400 transmission electron microscopy (TEM, JEOL, Tokyo, Japan), and particle size/distribution was analyzed by nano-flow cytometry (nanoFCM). The antibodies used in this study were listed in Additional Table 1.

Animals and SCI models

Seventy-five SPF-grade female Sprague-Dawley rats (200–220 g, 2 months old) were purchased from Laboratory Animal Center of Nantong University (license: SYXK(SU)2020-0029). All rats were randomized into SCI, TT (Treadmill training), Exo (MenSCs-Exo), and M (Multipurpose combination) groups ($n = 15$/group) by non-experimental personnel using the "random number table method" before proceeding with surgical modeling and subsequent experiments. All our subsequent evaluations were conducted using the "double-blind method" to ensure the objectivity and fairness of all data. The sample size was decided based on power calculation from the website: https://clincalc.com/stats/samplesize.aspx; continuous end point, two independent sample study. All animal procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at Nantong University (Approval number: IACUC20220820-018). The work has been reported in line with the ARRIVE guidelines 2.0. Rats were acclimated for 1 week under controlled conditions (22 ± 2 °C, 40–70% humidity). The rats were placed in an anesthesia induction chamber, and anesthesia was administered using a mixed gas of isoflurane and oxygen. Initial induction was performed with an isoflurane concentration of 4% for fast anesthesia. Once the rats were anesthetized, the isoflurane concentration was adjusted to 1.5% for maintenance until the end of the surgery. Their eyes were lubricated with eye ointment. Then, a T10 hemisection model was established by removing the T10 lamina, exposing the spinal cord, and transevering the right half with a scalpel. Sham rats underwent lamina removal without cord injury. Successful modeling was confirmed by hindlimb spasm and tail twitching. Post-surgery, rats were dwelled 2 per cage at 22 ± 2 °C, humidity of 40–70%, and 12/12-hour light/dark cycle. For analgesic purposes, sustained-release buprenorphine was administered subcutaneously at a dose of 0.05 mg/kg, while penicillin was given via intramuscular injection (i.m.) at a daily dosage of 20 IU for a 7-day period following the surgical procedure. And manual bladder massage was taken twice daily until autonomous micturition recovered. A summary of the study design is shown in Fig. 1.

The humane endpoint for euthanasia was established based on the following criteria: (1) inability to feed or drink independently, (2) complete tetraplegia, or (3) weight loss exceeding 20% of the initial body weight before cervical compression or decompression surgery. Rats satisfying any of these criteria were euthanized via an overdose of sodium pentobarbital (120 mg/kg, intraperitoneal injection).

MenSCs-exos treatment

Exo and M groups received tail vein injection of 200 μg MenSCs-Exo in 1mL PBS within 2 h post-SCI, repeated every 48 h (4 times total). Sham, SCI, and TT groups received PBS injections.

Body weight-supported treadmill training (BWSTT)

A custom BWSTT (patent: ZL200920235390.5) was used. Rats underwent 3 days of pre-injury adaptation; only trained rats were included. From day 3 post-SCI, TT and M groups received training: 6–20 m/min, 20 min/session, twice daily, 5 days/week for 4 weeks, with weight support reduced by 20–40% based on hindlimb function.

BBB score (Basso, Beattie, Bresnahan locomotor rating scale, BBB)

BBB scores were assessed pre-injury and on days 1, 3, 5, 7, 14, 21, 28 post-SCI by two trained observers, with mean values recorded (0 = paralysis, 21 = normal function).

Catwalk analysis

A CatWalk 9.0 system (Noldus) evaluated hindlimb coordination. Rats acclimated twice (15 min/session) before testing, with footprints recorded on a glass runway (≥ 3 passes/sample).

Tissue preparation

At 4 weeks post-intervention, rats were perfused with saline followed by 4% paraformaldehyde. Spinal cord segments (1 cm around the injury) were cryosectioned (10 μm) for histology/immunofluorescence or stored in liquid nitrogen (Western blot) or glutaraldehyde (electron microscopy).

Histological examination

Sagittal sections were stained with Masson's trichrome (fibrosis) and H&E (morphology, $n = 5$/group), dehydrated, cleared, and mounted for Image J analysis.

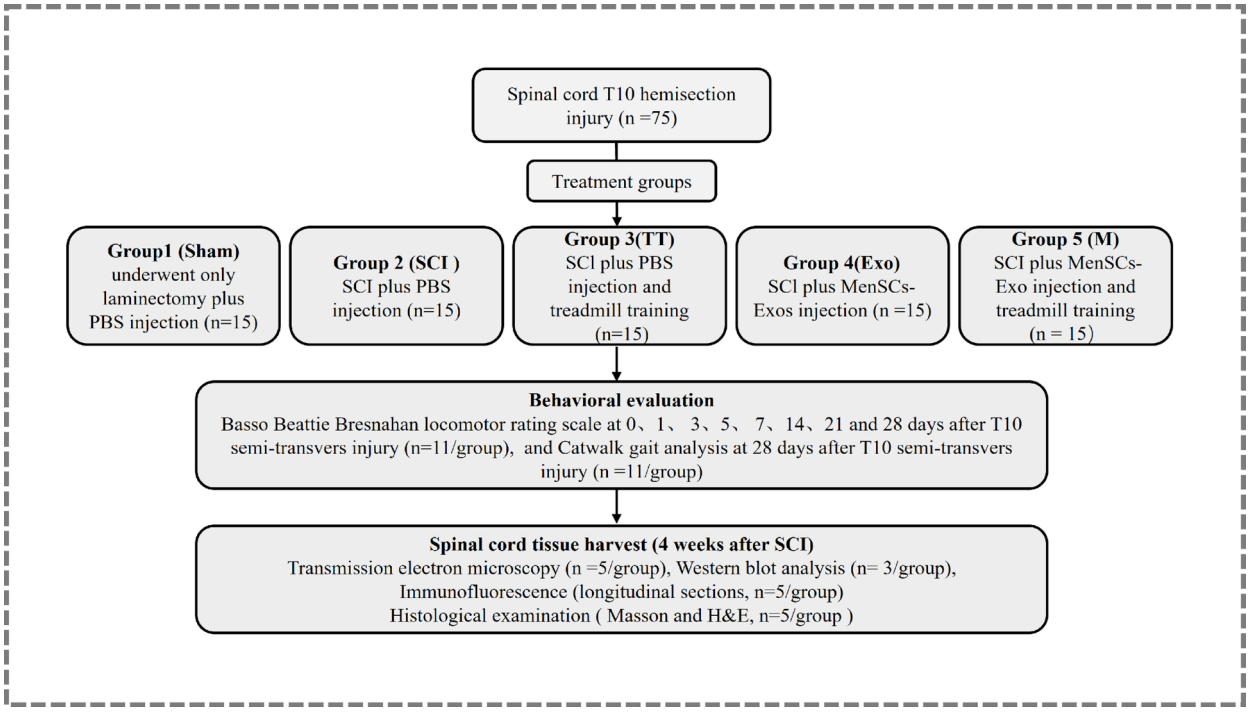


Fig. 1 Flowchart of the experiment

Transmission electron microscopy

Injury site tissues (1 mm^3) were fixed in 2.5% glutaraldehyde, post-fixed in osmium tetroxide, dehydrated, embedded in epoxy resin (Epon 812; Serva, Wetzlar, Germany), and ultrathin-sectioned (60–80 nm, EM KMR2 ultramicrotome; Leica) to uranyl acetate and lead citrate (MilliporeSigma) and examined by a JEM-1400 transmission electron microscopy (TEM, JEOL, Tokyo, Japan).

Western blot analysis

Spinal tissues were lysed in RIPA buffer, quantified by BCA, separated by 12% SDS-PAGE, and transferred to membranes. Primary antibodies (PI3K, p-Akt-S473, p-Akt-T308, β-catenin, SMA, Col1, Col3, LEF1, β-actin; Abcam, Cambridge, MA, USA. 1:1000) were incubated overnight, followed by HRP-conjugated secondary antibodies (Abcam, Cambridge, MA, USA. 1:2000). Bands were visualized by ECL (Solarbio, Beijing China) and quantified via Image J. The antibodies used in this study were listed in Additional Table 1.

Immunofluorescence

Sections were blocked with 0.5% Triton X-100, incubated with anti-Tuj1 or anti-MBP (Abcam, Cambridge, MA, USA. 1:1000) overnight, followed by fluorescent secondary antibodies and DAPI. Images were captured by fluorescence microscopy, and fluorescence intensity was analyzed by Nikon NIS software. The antibodies used in this study were listed in Additional Table 1. The region of interest (ROI) for histological analysis was specified as "the area 500 μm above and below the injury center", and standardized image acquisition was adopted (200× field of view, with 3 random regions sampled per specimen).

Statistical analysis

The data were statistically analyzed using SPSS 23.0 software and GraphPad Prism 8.0 software. Sample size was determined on the basis of the scientific significance and expected impact of the study. Referring to established statistical criteria, we used G*Power 3.1 to calculate that five animals per group would be required for a two-tailed α of 0.05 and a power (1-β) of 0.80. Because $n = 5$ per group, inter-group differences were analysed with the exact Kruskal–Wallis test. A resulting $P > 0.05$ simply indicates that the current sample lacked the power to detect a difference (power ≈ 30%); it does not demonstrate that no difference exists among the groups. Normally distributed measurement data were described as mean ± standard deviation (SD) ($\bar{x} \pm s$). Intergroup comparisons were performed using one-way analysis of variance (ANOVA), with Least Significant Difference (LSD) test for post-hoc pairwise comparisons. Skewed distributed measurement data were presented as median (interquartile range) [M(P25, P75)]. When heterogeneity of variance was observed, the Kruskal-Wallis H rank-sum test was used for group comparisons. Repeated measurement data across multiple groups were analyzed using

repeated measures analysis of variance. Statistical significance was defined as $P < 0.05$.

Results

MenSCs-exo characterization

Three conventional methods—transmission electron microscopy (TEM), Western blot (WB), and nanoFCM particle size analysis were used to characterize MenSCs-Exo. TEM observations revealed MenSCs-Exo with dark centers and bright edges, showing relatively uniform particles in a typical cup-shaped morphology (Fig. 2A). WB results confirmed positive expression of CD9, CD63, CD81, and TSG101 (specific exosomal markers) and negative expression of Calnexin in MenSCs-Exo (Fig. 2C). nanoFCM data demonstrated exosome particle sizes ranging from 47.8 nm to 162.4 nm, with an average size of 70.56 nm and a concentration of 5.04×10^9 particles/mL (Fig. 2B). Endocytosis experiments further showed that MenSCs-Exo could be internalized by SH-SY5Y cells (Fig. 2D). Thus, MenSCs-Exos were harvested, identified and exhibited biological activity.

MenSCs-exo combined with treadmill training promotes motor function recovery in SCI rats

Using the BBB scoring system and Catwalk gait analysis, the effects of different treatment approaches on hindlimb motor function recovery in SCI rats were evaluated. On the first day after model establishment, BBB assessments indicated complete paralysis of the right hindlimb in all SCI rats (BBB score = 0). Subsequently, following different treatments, the right hindlimb motor function of the rats showed varying degrees of recovery (Fig. 3A). Starting from day 7, the combined treatment group exhibited higher BBB scores than the SCI group, and these differences became even more pronounced by the fourth week post-SCI. Catwalk gait analysis visually displayed the trajectories of hindlimb movement under various treatments. At 28 days post-surgery, compared with sham-operated rats, the SCI group demonstrated pronounced hindlimb dragging and blurred footprints, whereas the combined treatment group showed clearer footprints. Although some dragging behavior remained at the fourth week, it was markedly improved compared with the other groups. Furthermore, footprint morphology, intensity, the distance between the two hind paws, and the relative footprint distance (Figs. 3B–F) were noticeably enhanced

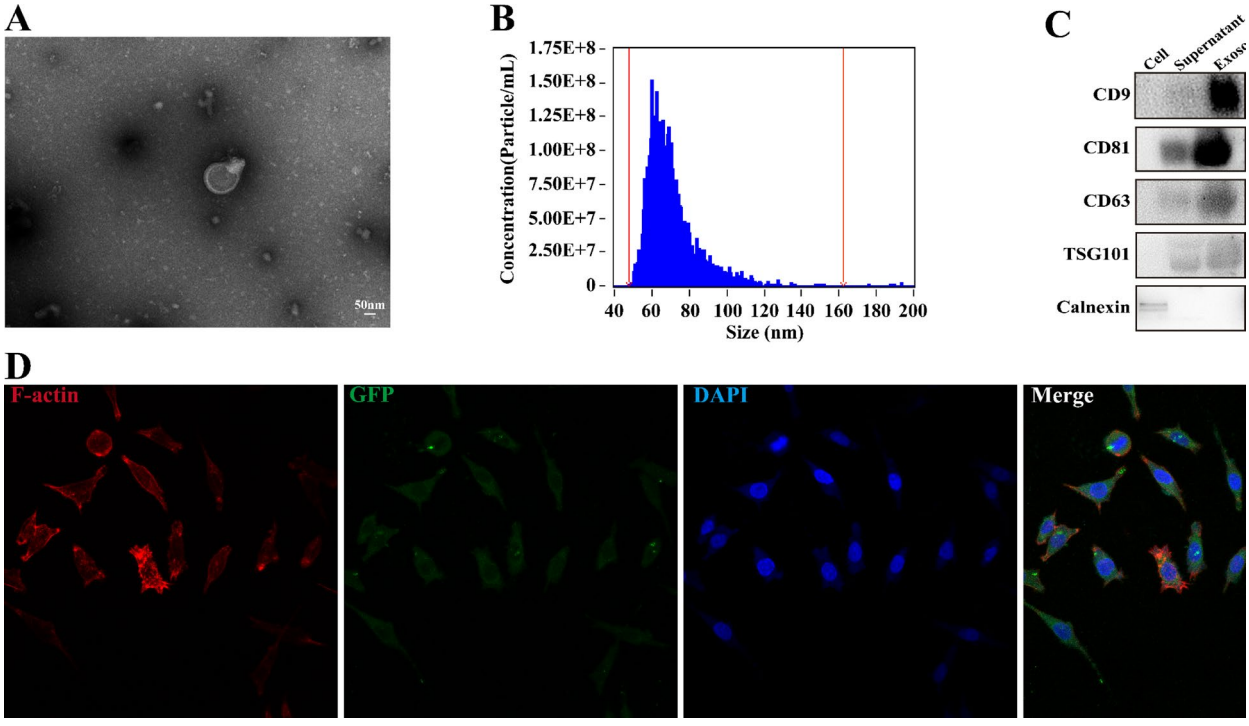


Fig. 2 Characterization of exosomes derived from menstrual blood stem cells. **A** The morphology of exosomes derived from menstrual blood stem cells (MenSCs-Exo) was observed by transmission electron microscopy (TEM). Acc. Voltage = 80.0 kV, Magnification = x20.0k, Scale bar = 500 nm. **B** The particle size and concentration of MenSCs-Exo were detected by nanoFCM particle size analysis. **C** Western blot was used to detect the expression of corresponding protein markers in cells (Cell), cell supernatant (Supernatant), and MenSCs-Exo. Full-length blots/gels are presented in Supplementary Fig. 1. **D** MenSCs-Exo were labeled with green fluorescent protein (GFP), the SH-SY5Y cells were labeled with phalloidin for filamentous actin (F-actin), and the cell nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI). It can be observed that MenSCs-Exo co-localized with SH-SY5Y cells, indicating that MenSCs-Exo can be internalized by neuronal cells. Scale bar = 50 μm

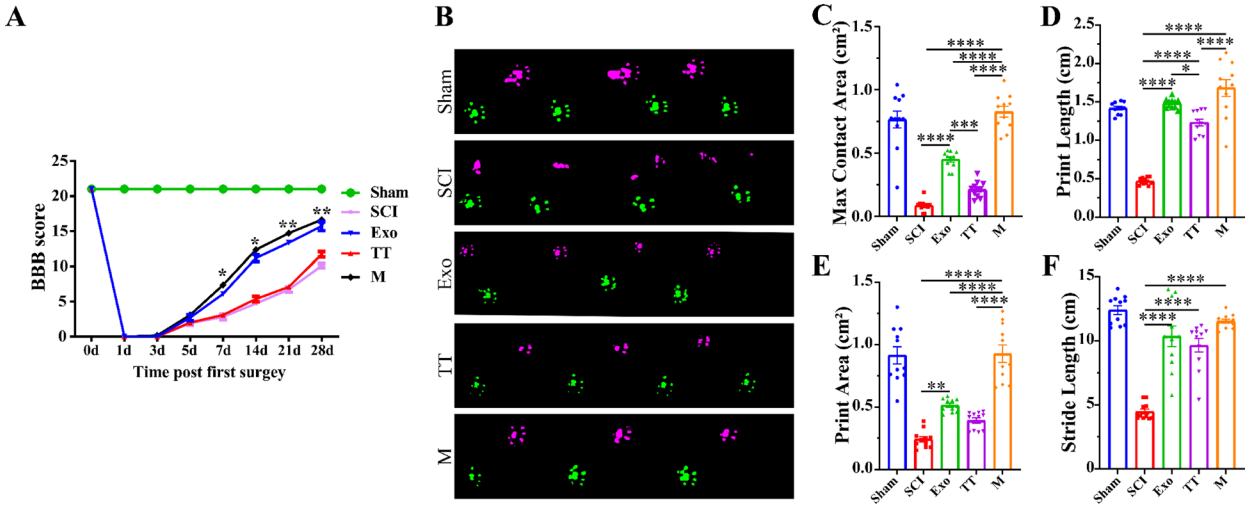


Fig. 3 Exosomes derived from menstrual blood stem cells combined with exercise training promote the recovery of motor function in SCI rats. **A** Basso, Beattie, and Bresnahan (BBB) scores of SCI rats at different time points (0, 1, 3, 5, 7, 14, 21 and 28 days) after surgery. At 28 days after surgery, the SCI rats underwent Catwalk gait analysis. The footprint morphology (**B**), Maximum contact area (**C**), footprint length (**D**), footprint area (**E**), and stride length (**F**). $n = 11$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

compared with the SCI group and surpassed those in the Exo group. Taken together, these findings suggest that, after combined treatment with exercise training and MenSCs, the degree of motor function recovery in SCI rats was significantly higher than that in rats receiving either MenSCs monotherapy or exercise training alone.

Combined therapy promotes histological repair of the injured spinal cord

We investigated the effects of combination therapy on the structural repair of injured spinal cord tissue. At the fourth week post-injury, hematoxylin and eosin (H&E) staining was employed to evaluate morphological changes in the damaged spinal cord (Fig. 4A and C). In the injury group, severe tissue damage and a large number of infiltrating inflammatory cells were observed, accompanied by extensive tissue cavities. In the Exo treatment group, weight-supported exercise training group, and combination therapy group, spinal cord integrity was improved. Although mild inflammatory infiltration was still present in the injured tissue, combination therapy significantly alleviated cellular shrinkage and fragmentation, reduced the number of inflammatory cells, and markedly mitigated spinal cord cavity formation compared with the other groups (Fig. 4C).

Combined therapy reduces local fibrosis in injured spinal cord

To determine the effect of exosomes derived from menstrual blood stem cells combined with exercise training on the degree of fibrosis in the injured spinal cord tissue of rats after SCI, spinal cord tissue was harvested at 28 days post-operation, cryosectioned for Masson staining, and analyzed by WB for fibrosis-related protein expression. Masson staining revealed reduced collagen volume fraction (CVF) in the SCI region of the combination treatment group compared with other injury groups (Fig. 4B and D). Western blot analyses indicated that COL1, COL3, and α-SMA expression in SCI rats receiving the combined therapy was lower than that observed in the injury group and single-factor treatment groups (Fig. 5A–D). These findings also suggest that exosomes derived from menstrual blood stem cells, together with exercise training, significantly alleviate fibrosis in the SCI region compared with the injury group and single-factor treatments.

Combined therapy promotes neuronal axon regeneration in SCI rats

To determine the effect of combining MenSCs-Exo with exercise training on neuronal axon regeneration following SCI, spinal cord tissue was collected at 28 days post-injury, cryosectioned, and subjected to immunofluorescence (Fig. 6A, green for GFAP, red for Tuj1, blue for DAPI). Injury in the cord elevated the reaction of astrocytes as revealed by the enhanced expression of GFAP while decreased by the combinatory treatments (Fig. 6B). In the SCI group, neuronal axons at the injury site were disrupted, showing no evidence of axonal regrowth. Although partial axonal regeneration was observed in the MenSCs-Exo group, the number of Tuj1-positive axonal fibers was limited and disorganized. In contrast, the combination therapy group displayed a stronger Tuj1 signal than the other injured groups, indicating pronounced axonal regeneration with orderly arrangement (Fig. 6C).

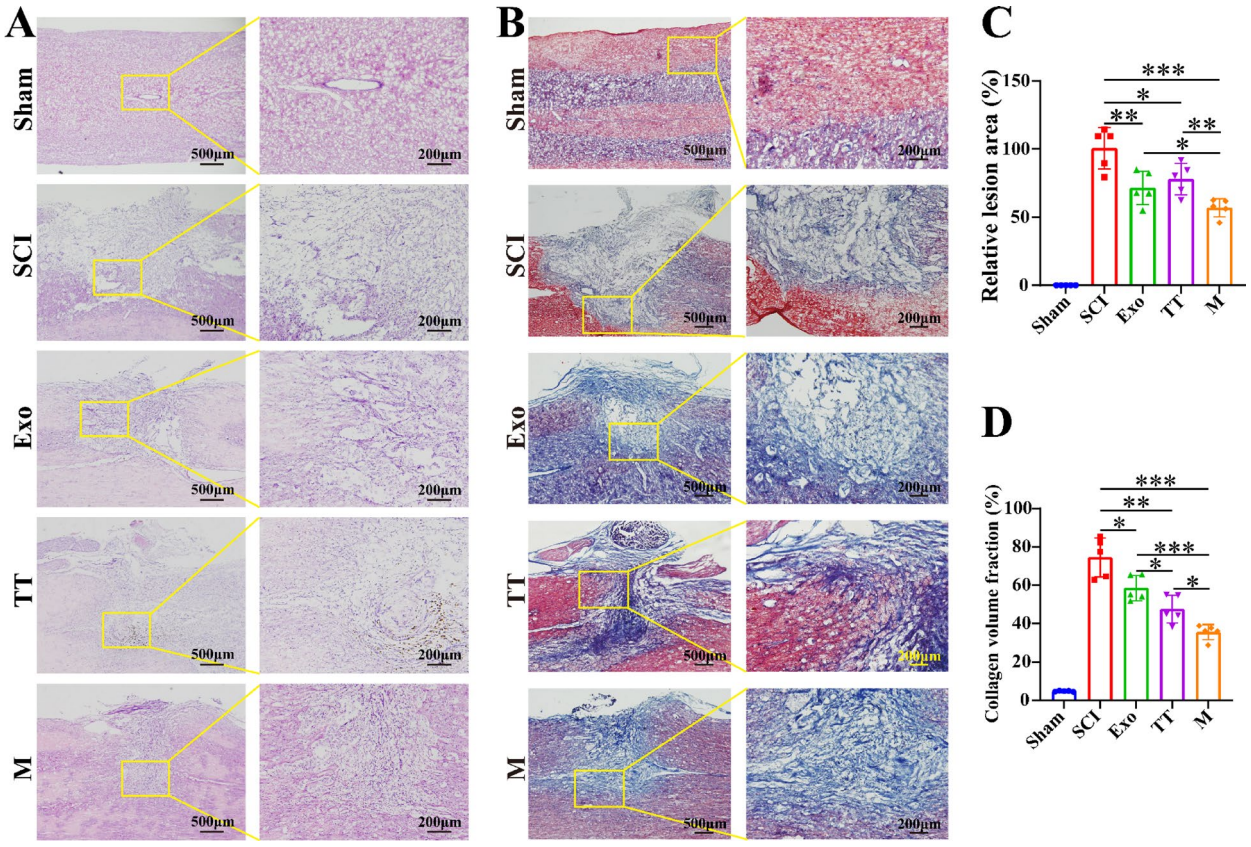


Fig. 4 Exosomes derived from menstrual blood stem cells combined with exercise training promote the repair of local tissues at the injury site and the improvement of the degree of fibrosis in SCI rats. **A** Hematoxylin and eosin (H&E) staining was used to evaluate the changes in tissue morphology at the spinal cord injury site in each group of SCI rats 28 days after surgery. The scale bars are 500 μm and 200 μm. **B** Masson's trichrome staining was used to evaluate the fibrosis at the spinal cord injury site in each group of SCI rats 28 days after surgery. The scale bars are 500 μm and 200 μm. ($n=5$). **C** Statistical analysis of the relative lesion area in Figure (A) ($n=5$). **D** Statistical analysis of the relative lesion area in Figure (B) ($n=5$). *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$

Combined therapy promotes myelin reconstruction in injured spinal cord

To further evaluate remyelination of residual axons and examine the effect of combining menstrual blood stem cell-derived exosomes with exercise training on myelin regeneration after SCI, spinal cord tissue was similarly collected at 28 days post-injury, cryosectioned, and examined by immunofluorescence (Fig. 7A, green for GFAP, red for MBP, blue for DAPI). Same results of astrocytes reaction were observed as illustrate in Figs. 6B, 7B. The SCI group displayed extensive demyelination, with interrupted myelin sheaths at the injury site, partial or complete lamellar separation, and a lack of normal myelin structure. Although some myelin regeneration was noted in the MenSCs-Exo group, the number of MBP-positive myelin sheaths was limited and arranged without clear organization. In contrast, the combination therapy group showed stronger MBP fluorescence, indicating notable myelin regeneration in an orderly pattern (Fig. 7C).

Furthermore, at 28 days post-SCI, electron microscopy was used to analyze myelin ultrastructure in each group (Fig. 8). The control group showed regular alternating light and dark "track-like" dense lamellar structures with no shrinkage, tightly arranged myelin, full nuclei, abundant cytoplasm, no demyelination, and uniform axonal electron density without vacuoles. In the SCI group, there was significant myelin swelling, irregular morphology, loose and layered lamellae, and partially fused or missing myelin, along with axonal atrophy and degeneration, blurred structural borders, vacuole formation, lowest myelin sheath thickness, and even axonal disappearance. Compared with the SCI and single-treatment groups, the combination therapy group exhibited markedly less loosening and loss of myelin lamellae in the lesion area, improved myelin sheath thickness, and an increased number of myelin sheaths on residual axons, while axonal atrophy and vacuolation were substantially alleviated (Fig. 8F).

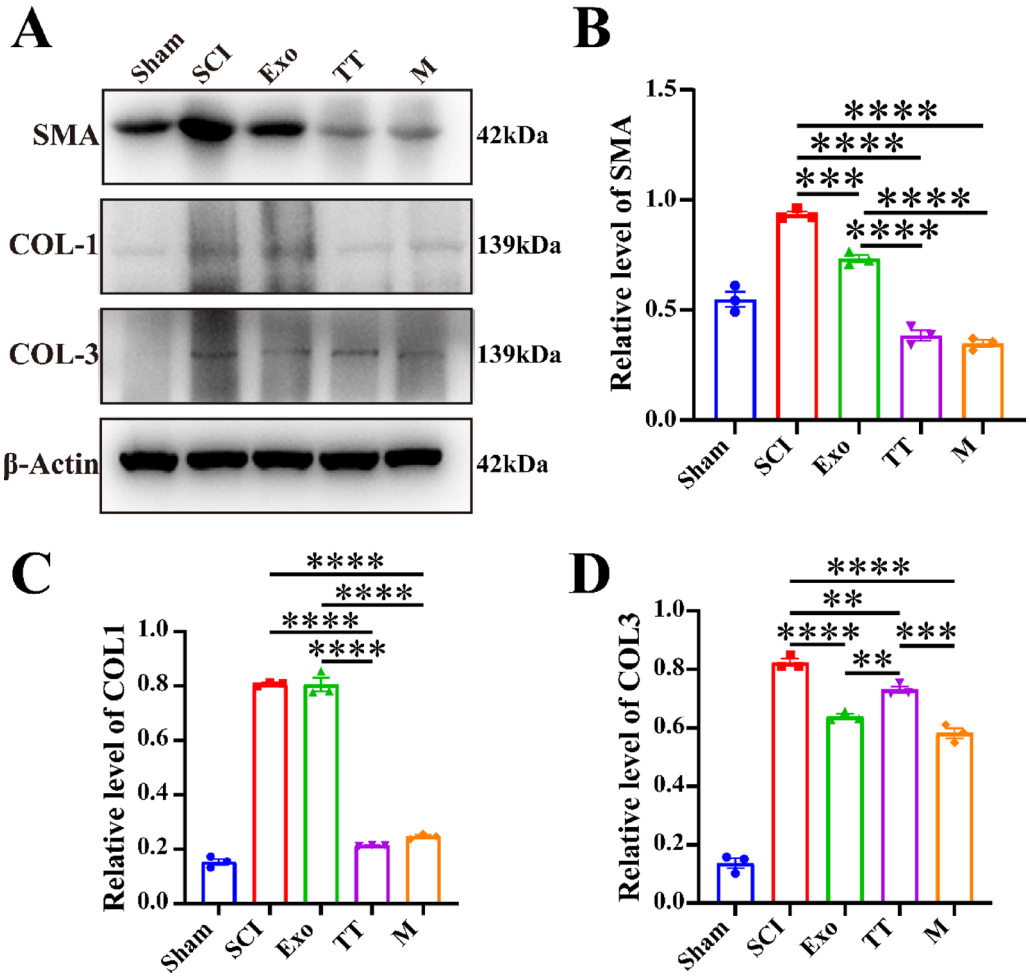


Fig. 5 Exosomes derived from menstrual blood stem cells combined with exercise training promote the improvement of the degree of fibrosis in the local tissues at the injury site in rats with spinal cord injury (SCI). **A** Western blot was used to detect the expression levels of the fibrotic proteins α-smooth muscle actin (α-SMA), collagen type I (COL1), and collagen type III (COL3) at the spinal cord injury site in each group of SCI rats 28 days after surgery. **B** Quantitative statistical graph of the α-SMA protein. **C** Quantitative statistics of the COL1 protein. **D** Quantitative statistics of the COL3 protein. $n=3$, the exact Kruskal–Wallis test was used to compare inter-group differences. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. Full-length blots/gels are presented in Supplementary Fig. 2

Combined therapy activates the PI3K/Akt pathway to synergistically enhance SCI recovery

To further confirm whether the combination of menstrual blood stem cell-derived exosome therapy and exercise training promotes SCI recovery by activating the PI3K/Akt pathway, we performed Western blot analyses of key molecules in this pathway (Fig. 9). The results showed that, compared with the injury-only group, combination therapy significantly increased the expression levels of phosphorylated Akt and PI3K (Akt-S473: $P<0.05$; Akt-T308: $P<0.001$; PI3K: $P<0.05$; Fig. 9A-G). Meanwhile, combination therapy markedly elevated β-catenin and LEF1 levels, although LEF1 expression was highest in the exosome therapy group and remained largely unchanged compared with that in the exercise training group (Fig. 9F–G). These findings indicate that MenSCs-Exo combined with exercise training can activate the PI3K/Akt and β-catenin/LEF1 signaling pathways.

Discussion

General overview

Spinal cord injury (SCI) is a severe central nervous system disorder with high morbidity and disability, causing sensorimotor, urinary, and digestive dysfunction. Neural repair therapies, focusing on enhancing plasticity and synaptic remodeling, are central to treatment. Combinatorial strategies outperform monotherapies. Mesenchymal stem cells and exosomes exhibit therapeutic potential by inhibiting neuronal apoptosis and promoting neurogenesis/synaptic regeneration, improving post-SCI recovery [20]. Body weight-supported treadmill training (BWSTT) is a neurorehabilitation therapy using suspension to reduce limb load, aiding ambulation in SCI

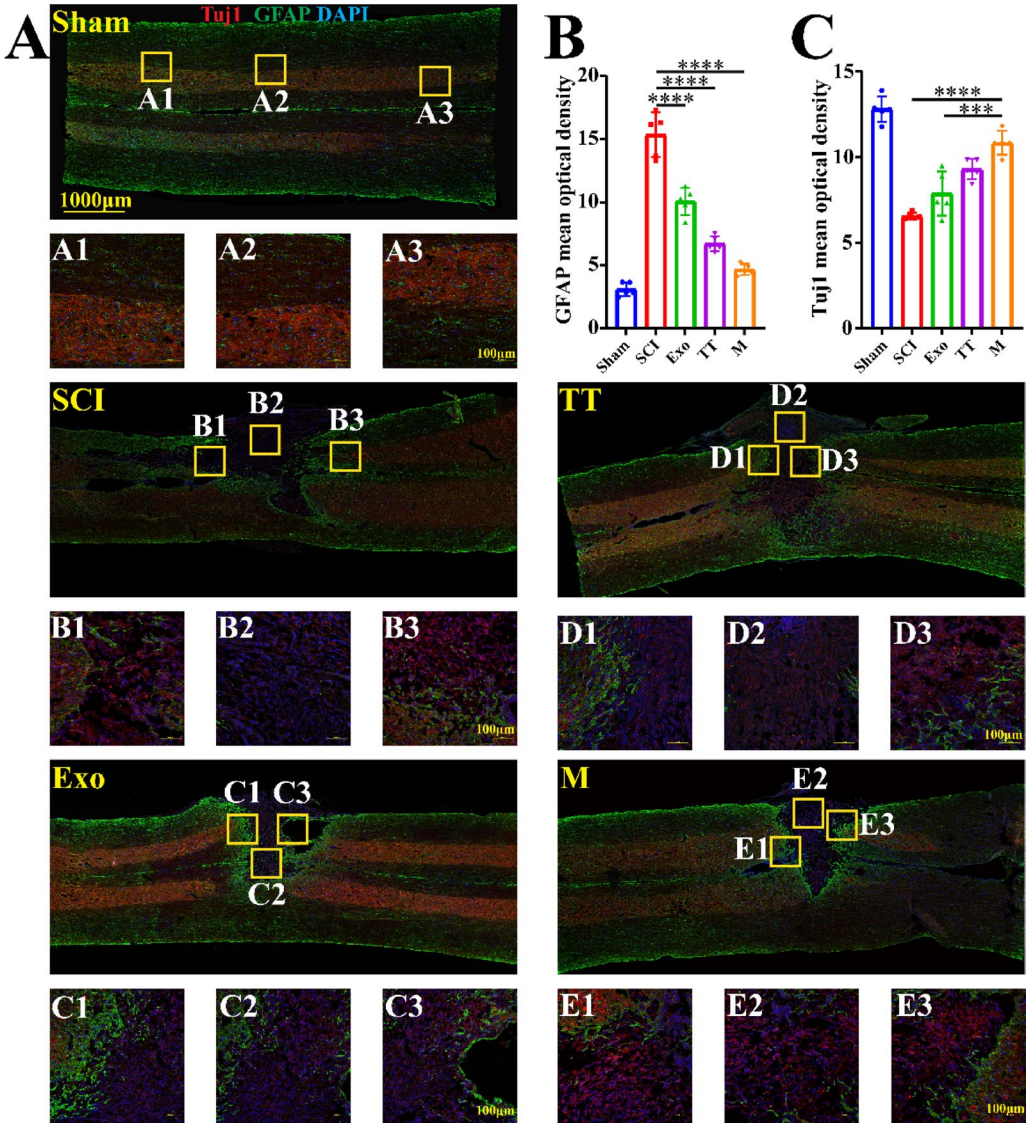


Fig. 6 Exosomes derived from menstrual blood stem cells combined with exercise training promote nerve regeneration in SCI rats. **A** The spinal cord tissues of rats in each group were collected 28 days after SCI. After frozen sectioning, immunofluorescence was performed to observe the expression of Tuj1 at the injury site: Tuj1 (red), glial fibrillary acidic protein (GFAP) (green), 4′,6-diamidino-2-phenylindole (DAPI) (blue), and the scale bars are 1000 μm and 100 μm. **B** Statistical analysis of the fluorescence intensity of GFAP in Figure A. **C** Statistical analysis of the fluorescence intensity of Tuj1 in Figure A. $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

patients [21]. Isolated use has limited efficacy; combinatorial approaches are critical for optimizing neural activation and functional recovery.

Prior research demonstrated that menstrual blood-derived mesenchymal stem cells (MenSCs) promote hindlimb motor recovery in SCI rats [10], while BWSTT enhances brain-derived neurotrophic factor (BDNF) and tropomyosin-related kinase B (TrkB) expression in the distal spinal cord, thus improving motor function [22]. Further investigations revealed combined MenSCs therapy and treadmill training enhanced graft survival, differentiation, proliferation, and BDNF secretion, thereby improving spinal cord tissue architecture and motor recovery [10]. Building upon the promising therapeutic profile of MSC-derived exosomes (MenSCs-Exo), this study examined the efficacy of combined MenSCs-Exo treatment and sensorimotor training on neural regeneration and functional improvement. Our results indicated that MenSCs-Exo combined with BWSTT synergistically promoted hindlimb motor function recovery by enhancing axonal and myelin regeneration, synaptic plasticity, inhibiting fibrosis, and neuroprotection, notably through activation of the PI3K/Akt signaling pathway.

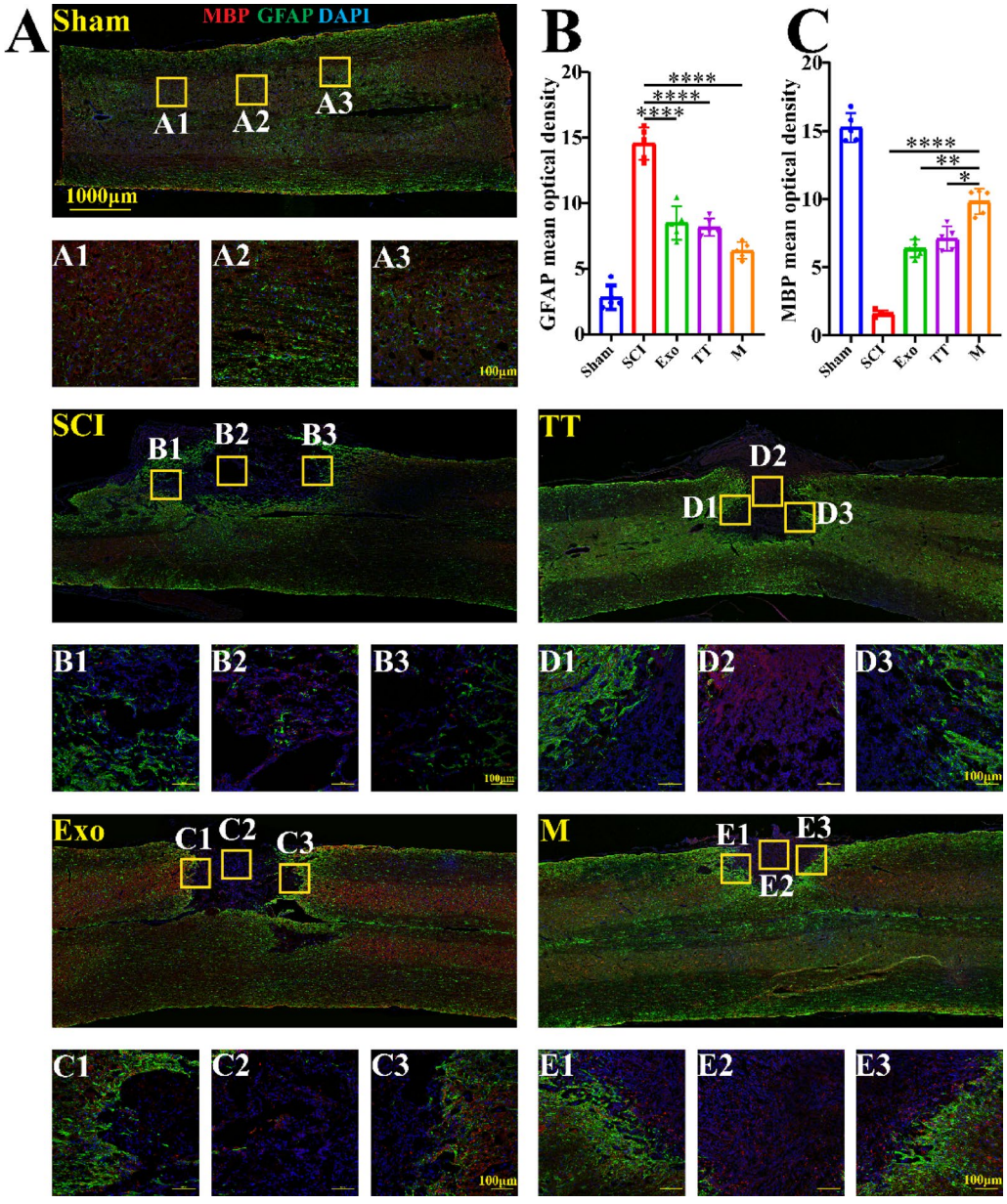


Fig. 7 Exosomes derived from menstrual blood stem cells combined with exercise training promote the regeneration of damaged myelin sheaths in SCI rats. **A** The spinal cord tissues of rats in each group were collected 28 days after SCI. After frozen sectioning, immunofluorescence examination was carried out to evaluate the expression of myelin basic protein (MBP) at the injury site: myelin basic protein MBP (red), glial fibrillary acidic protein (GFAP) (green), 4′,6-diamidino-2-phenylindole (DAPI) (blue), and the scale bars are 1000 μm and 100 μm. **B** Statistical analysis of the fluorescence intensity of GFAP in Figure A. **C** Statistical analysis of the fluorescence intensity of MBP in Figure A. $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

Effects of MenSCs-exo on functional recovery after SCI

SCI represents a multifaceted pathological cascade characterized by markedly limited intrinsic regenerative capacity, highlighting stem cell-based therapies as a regenerative medicine frontier for neural repair. Recent evidence underscores the beneficial role of stem cell-derived exosomes-paracrine effectors of therapeutic MSCs-in reducing neuronal apoptosis, attenuating inflammation, promoting angiogenesis, and facilitating behavioral improvement post-SCI. Compared with direct cellular transplantation [23], MSC-derived exosomes possess distinctive advantages, including minimal risk of tumorigenicity, thrombosis, or malignant transformation, capacity to traverse the blood-brain barrier, and low immunogenicity facilitating allogeneic applications [24, 25]. Animal studies suggest that exosome treatments confer comparable or superior therapeutic benefits relative to intact MSCs, notably in anti-inflammatory and anti-scarring effects [26]. Human umbilical cord MSC-derived exosomes encapsulated in gelatin scaffolds promoted

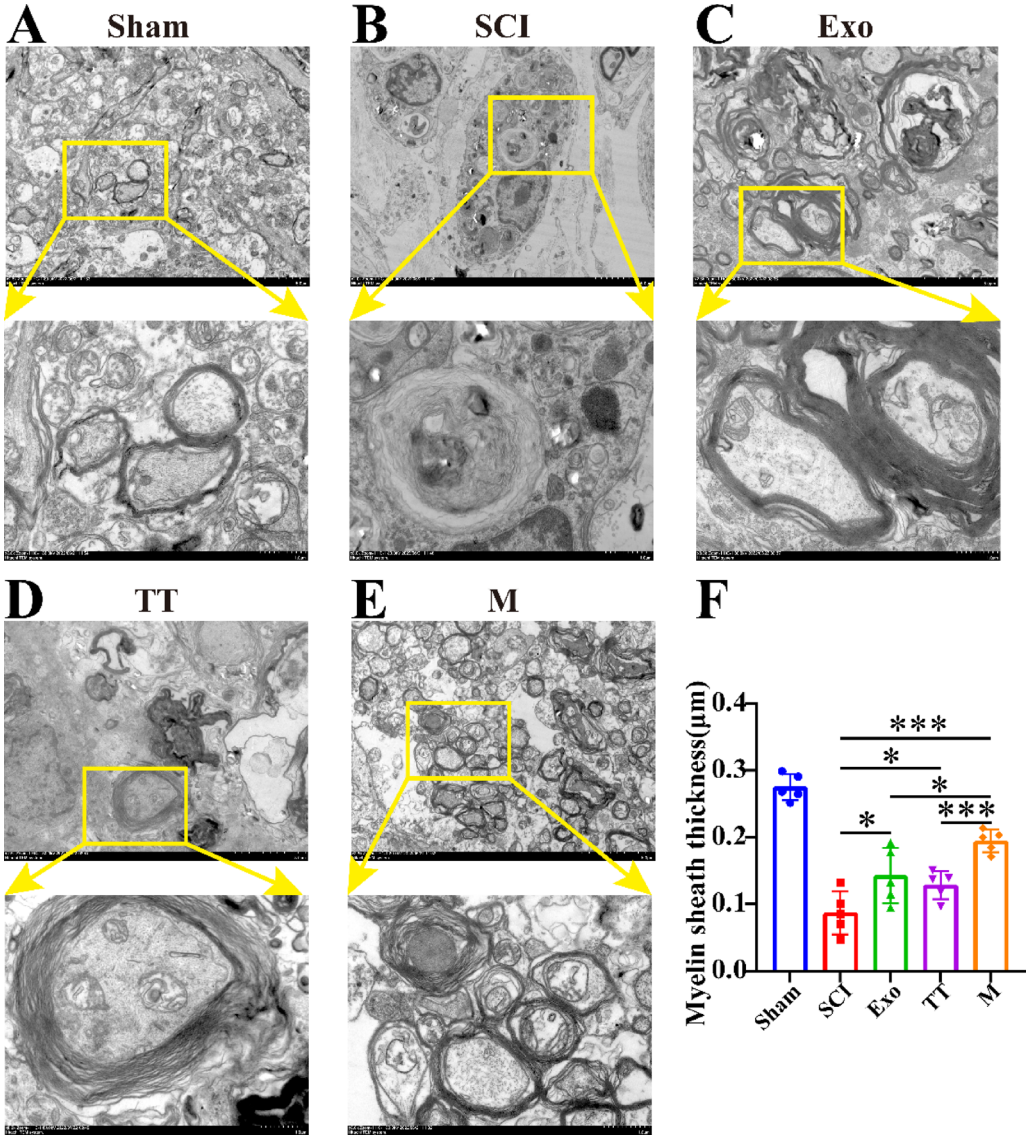


Fig. 8 Exosomes derived from menstrual blood stem cells combined with exercise training promote the improvement of the structure of damaged myelin sheaths in SCI rats. The spinal cord tissues of rats in each group were collected 28 days after SCI, and the ultrastructure of myelin sheaths in rats of different groups was analyzed by electron microscopy. The scale bars are 5.0 μm and 1.0 μm. **A** Sham group, **B** SCI group, **C** Exo group, **D** TT group, **E** M group. **F** Statistical analysis of the myelin sheath thickness in all groups. $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

locomotor improvement in SCI models through neuroregenerative, myelinating, anti-inflammatory, and anti-apoptotic mechanisms [27]. In our study, MenSCs-Exo monotherapy similarly facilitated spinal cord structural repair, axonal and myelin regeneration, and consequently improved functional recovery, substantiating MenSCs-Exo's robust reparative potential in SCI.

Effects of body weight-supported treadmill training on functional recovery after SCI

BWSTT, conceptualized by Finch based on central pattern generator theory, involves limb suspension to reduce gravitational loading, facilitating locomotor training and enhancing walking function [28]. Previous findings indicate BWSTT alleviates SCI-induced motor neuron hyperexcitability and spasticity by modulating TrkB signaling and increasing spinal expression of GAD-65, GAD-67, and KCC2 [22]. Nonetheless, the current study observed limited therapeutic efficacy with BWSTT alone, reflected by non-significant differences in BBB scores compared with untreated controls. We attribute this outcome primarily to intervention timing, training duration, and intensity. Specifically, ultra-early initiation (day 3 post-injury) might have exacerbated spinal instability and impaired resolution of edema and hemorrhage [29]. Furthermore, short-duration (4-week) training likely lacked sufficient intensity and longevity required to yield meaningful improvement, as evidenced by existing literature

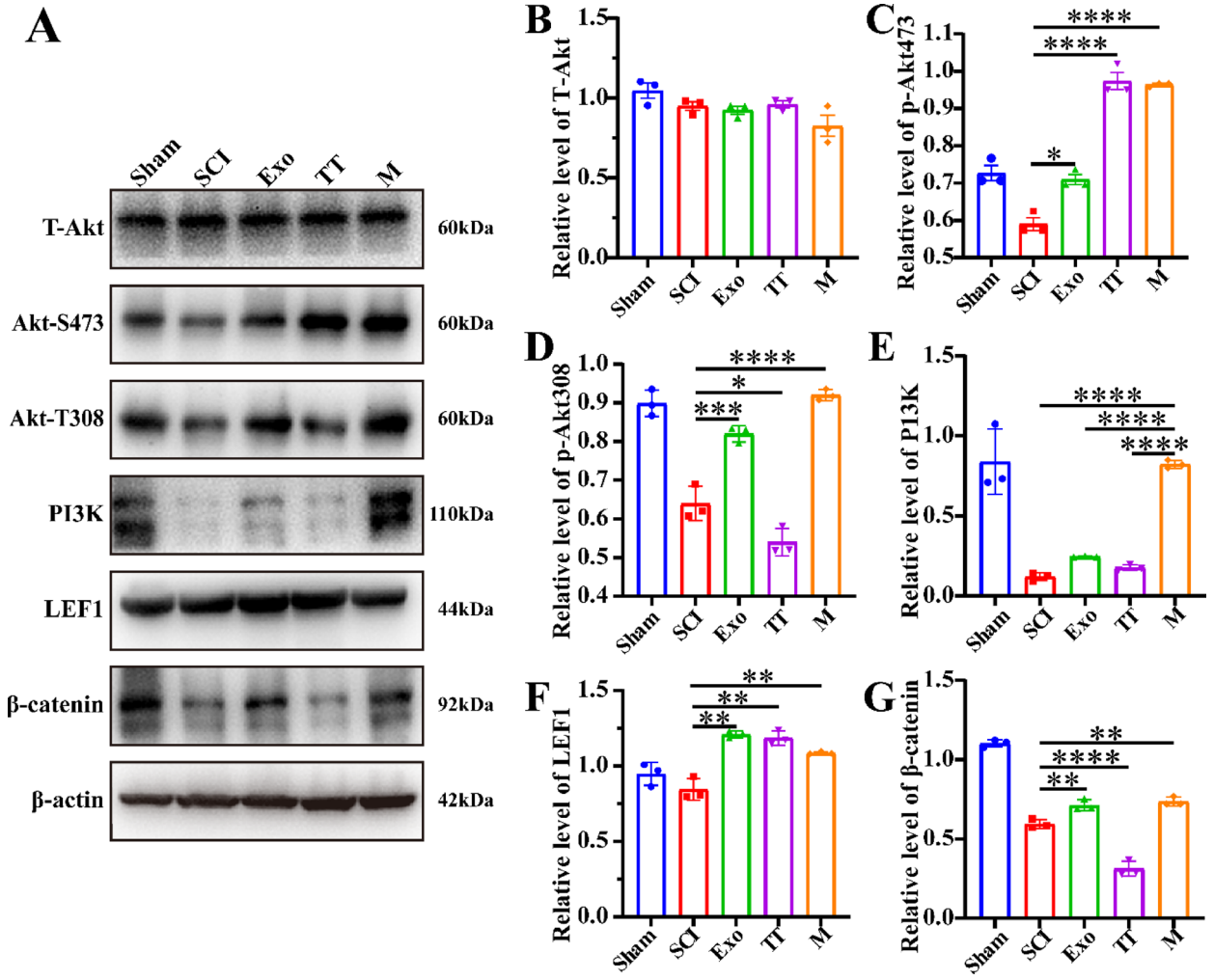


Fig. 9 Exosomes derived from menstrual blood stem cells combined with exercise training activate the PI3K/Akt pathway. **A** Western blot was performed on the spinal cord tissues of rats in each group 28 days after SCI to detect the expression of proteins such as total Akt (T-Akt), phosphorylated Akt at serine 473 (Akt-S473), phosphorylated Akt at threonine 308 (Akt-T308), phosphatidylinositol 3-kinase (PI3K), β-catenin, and lymphoid enhancer-binding factor 1 (LEF1). Quantitative statistics of the T-Akt protein (**B**), Akt-S473 protein (**C**), Akt-T308 protein (**D**), PI3K protein (**E**), LEF1 protein (**F**), and β-catenin protein (**G**). $n = 3$, the exact Kruskal–Wallis test was used to compare inter-group differences. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Full-length blots/gels are presented in Supplementary Fig. 3

demonstrating optimal therapeutic effects with medium-to-long-term (9–16 weeks) moderate-to-high-intensity protocols [30, 31]. Therefore, future studies should emphasize initiating appropriately timed, prolonged, and sufficiently intensive BWSTT protocols post-SCI.

Effects of combined MenSCs-Exo and treadmill training on functional recovery after SCI

Influence on fibrosis and tissue repair

Scar formation after SCI arises from activated glial cells and fibroblasts, causing excessive collagen deposition and scar tissue formation [32]. Containing inhibitory molecules like collagen fibers and proteoglycans, scars restrict axon regeneration and impair motor/sensory recovery, inhibiting scarring promotes neural regeneration [33, 34]. SCI scars include fibrotic (from pericytes/extracellular matrix) and glial (from astrocytes) types [35, 36]. Collagens, rarely expressed in healthy spinal cord, play dual roles: early scars limit inflammation and support axon growth, while dense scars hinder regeneration [37–39]. Type I collagen drives glial scarring and fibrosis [33, 40, 41]; Type III collagen aids early scar remodeling. High α-smooth muscle actin (SMA) correlates with severe fibrosis [42]. Our study shows menstrual blood stem cell-derived exosomes combined with treadmill training reduce type I/III collagen and SMA expression at 28 days post-injury, decreasing fibrosis and facilitating axon regeneration [43]. Our data demonstrated MenSCs-Exo plus BWSTT significantly suppressed these fibrotic markers, thus ameliorating scarring and fostering a conducive regenerative microenvironment.

Influence on myelin regeneration

Myelin loss following SCI disrupts normal neuronal transmission, contributing to functional deficits [44]. Targeting myelin regeneration [45], specifically through promoting myelin basic protein (MBP) expression [46], is crucial [47]. Our combined therapy markedly improved myelin structural integrity, MBP intensity, and compactness, enhancing axonal support and conduction velocity, thereby significantly improving neurological recovery.

Influence on axonal regeneration

Axonal regrowth post-SCI faces numerous hurdles, including inhibitory scar environments, inflammatory mediators, and limited intrinsic neuronal regenerative capacity [48, 49]. Here, combinatorial therapy resulted in significantly increased axonal regeneration, evidenced by enhanced Tuj1 immunofluorescence and improved spatial organization compared to monotherapy and untreated groups, correlating positively with functional recovery [50]. This underscores combinatorial treatment's distinct advantage in promoting efficient axonal regeneration.

Activation of PI3K/Akt pathway in functional recovery

The PI3K/Akt pathway critically regulates cellular survival, proliferation, metabolism, and regeneration. Activation of PI3K/Akt signaling facilitates neural regeneration and attenuates inflammation post-SC [51] I. In this study, combinatorial MenSCs-Exo and treadmill training activated the PI3K/Akt cascade [52, 53], enhancing neuronal survival [54], facilitating repair processes [55], and optimizing neurological outcomes [56]. Additionally, Wnt/β-catenin signaling [57, 58], activated concurrently, contributed significantly to the observed reparative and regenerative effects, further supporting the therapeutic efficacy of this multimodal intervention.

Conclusion and prospects

Primary therapeutic objectives for SCI encompass attenuating secondary injury, promoting axonal regeneration, facilitating remyelination, and enhancing neuroplasticity. Our multimodal analysis conclusively demonstrated that MenSCs-Exo monotherapy facilitated functional improvement and spinal cord structural repair. Importantly, combinatorial therapy significantly outperformed individual interventions, reducing cavitation and fibrosis, enhancing neuronal survival, and markedly improving axon-myelin regeneration. These combined mechanisms yielded superior motor recovery outcomes compared to monotherapies alone. Consequently, the integrative strategy involving MenSCs-Exo administration and BWSTT emerges as a promising clinical therapeutic model warranting rigorous translational research for clinical application. Further studies should optimize therapeutic timing, dosage, and training paradigms, ultimately enabling successful clinical translation for comprehensive SCI neurorehabilitation.

Abbreviations

SCI	Spinal cord injury
MenSCs	Menstrual blood-derived mesenchymal stem cells
MenSCs-Exo	Mesenchymal stem cell-derived exosomes
YLD	Years lived with disability
MSCs	Mesenchymal stem cells
PI3K	Phosphatidylinositol 3-kinase
TT	Treadmill training
TEM	Transmission electron microscopy
BWSTT	Body Weight-Supported Treadmill Training
BBB score	Basso, Beattie, Bresnahan locomotor rating scale
H&E	Hematoxylin and eosin staining
BDNF	Brain-derived neurotrophic factor
TrkB	Tropomyosin-related kinase B
SMA	Smooth muscle actin
MBP	Myelin basic protein

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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The authors declare that they have not use AI-generated work in this manuscript.

Author contributions

LJQ, CMD, YYS and QHW conceived the idea and designed the research studies. YHS, LYZ, ZLL and WWJ conducted the experiments. LJQ, YXP, JQD, JXC and JYL participated in SCI model preparation and data analysis. SYC, YYS, HQS and CMD acquired and analyzed the data. LJQ and YYS drafted the initial manuscript. LJQ, CMD and QHW reviewed and revised the manuscript. All authors approved the final version of manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All animal procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at Laboratory Animal Center of Nantong University. Project title: Exosomes derived from Menstrual blood-derived mesenchymal stem cells (MenSCs-Exo) and Treadmill Training restore motor function following spinal cord injury. Project number: IACUC20220820-018. Approval date: August 20, 2022. For human Menstrual blood samples, the patients or their guardians provided written informed consent for participation in the study and the use of samples. All the menstrual blood samples' experiments were carried out according to the guidelines of the Ethics Committee of the Affiliated Nantong Hospital 3 of Nantong University. Study title: MenSCs-Exo loaded GelMA hydrogel promotes functional recovery

via miR-421/MAGED1/PI3K-Akt axis. Approval number: EK2023111. Approval date: October 6, 2023.

Consent for publication
Not applicable.

Competing interests
All authors declare no competing interests.

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