Clinics in Surgery

Ultra-Short-Wave Therapy Combined with Bone Marrow Mesenchymal Stem Cell Transplantation Modulates the Inflammatory Response after Spinal Cord Injury

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Abstract

Objective: To observe the effect of low-dose Ultra-Short-Wave (USW) therapy combined with Bone Marrow Mesenchymal Stem Cell (BMSC) transplantation on regulating macrophage polarization after Spinal Cord Injury (SCI) to reduce the early inflammatory response, and to discuss its potential mechanism.

Methods: Super-Paramagnetic Iron Oxide Nanoparticles (SPIONs) were used to label BMSC and observed the labeling effect through Prussian blue staining. About 120 Female Sprague-Dawley rats were randomly divided into five groups: sham-operated, control, USW, BMSC, and USW+BMSC that were performed to spinal cord contusion. Rats in the BMSC and USW+BMSC groups received BMSC transplantation, while those in the USW and USW+BMSC were exposed to USW radiation. Basso-Beattie-Bresnahan (BBB) tests were operated before the surgery and at 1-day, 3-day and 7-day intervals after SCI. The expressions of the ED1 and inducible nitric oxide synthase, Arginase1, markers of activated macrophages in the damage area were assessed with immunohistochemistry.

Results: Four weeks after SCI, BMSCs survived and had irregular polymorphic forms. Super-Paramagnetic Iron Oxide Nanoparticles (SPIONs) were also visible in the cytoplasm. Compared with the BMSC group, cell survival was better in the USW+BMSC group. Seven days after SCI, rats in the USW and BMSC+USW groups had better Basso-Beattie-Bresnahan scales cores compared with the control group. Compared with the USW and BMSC groups, Ectodermal Dysplasia 1 (ED-1) expression was decreased in the USW+BMSCs group at 1 week after surgery. Expression of inducible nitric oxide synthase in the BMSC and USW+BMSC groups was lower than that in the USW group 3 days after SCI. Arginase 1 (Arg 1) expression in the BMSC and USW+BMSC groups was higher than that in the control group.

Conclusion: Low-dose USW therapy with BMSC transplantation can modulate the inflammatory response after SCI, and may be a key component of a combined approach to accelerate nerve regeneration and functional recovery. The correct dose of SPIONs to label BMSCs can be used for long-term observation after transplantation into damaged areas.

Keywords: Bone marrow mesenchymal stem cells; Macrophage polarization; Spinal cord injury; Superparamagnetic iron oxide nanoparticles; Ultra-short-wave therapy

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Introduction

Spinal Cord Injury (SCI) is a devastating event and it often leads to severe and permanent disability because of the limited prognosis of sensory and motor function due to the detrimental microenvironment formed at the injury site [1]. SCI can be divided into primary and secondary injury according to its pathological changes [2]. A series of pathological processes occurs following SCI: ischemia, inflammation, tissue edema, and excitotoxic damage, which aggravate the primary damage, thus affecting functional recovery, neural repair and regeneration [3-5]. The inflammatory response is one of the most notable features and persists throughout the period of SCI; its main features are increased vascular permeability and extravasation of neutrophils and macrophages into the tissues [6]. Local inflammation can promote the formation of cavum and a glial scar after SCI, thus impeding the damage repair process [7,8]. Macrophages (activated microglia and infiltrated monocytes) are involved in initiation and resolution of inflammation after SCI, and most

macrophages polarize into an inflammatory (M1) phenotype and only a small number into an anti-inflammatory (M2) phenotype. M1 macrophages can directly induce neuronal death and demyelination and inhibit axonal growth, while M2 macrophages can exert tissue repair and promote axonal regeneration after SCI [9,10]. Thus, macrophages as a pivotal cellular component in inflammation play a double-edged role during the process of damage repair. Methods to control the polarization of M1 to M2 macrophages are of great interest for reducing early inflammation in SCI.

Ultra-Short-Wave (USW) therapy has been widely used in clinical treatment because of its safety and effectiveness. USWs promote Schwann cell proliferation and nerve axon regeneration, and improve the microenvironment by promoting blood circulation and nutrition metabolism, which alleviates inflammation and reduces tissue edema [11]. In our previous study we demonstrated that USW therapy reduced lesion area and promoted recovery of hind limb movement in rats after SCI [12].

Stem cell transplantation is a promising method for treatment of SCI. It is reported that Bone Marrow Mesenchymal Stem Cells (BMSCs) are an ideal source of stem cells for transplantation because of their abundance, high self-renewal capacity, pluripotency, and weak immunogenicity [13]. BMSCs can secrete various neurotrophins to promote axonal regeneration, differentiate into neural cells to replace necrotic or apoptotic ones, and reconstruct damaged neural connections [14]. It has been shown that, in a worst case scenario after SCI, transplanted stem cells would be more inclined to differentiate into neural glial cells rather than neurons, which is unfavorable to functional recovery and nerve reconstruction [15]. Therefore, providing a suitable environment for transplanted stem cells is essential to achieve a therapeutic aim. Macrophages are recognized as important immune cells involved in the inflammatory response after SCI, and various cytokines secreted by macrophages participate in the regulation of the injured microenvironment [16]. Little is known about the synergistic effects of USW therapy combined with BMSC transplantation on directing macrophage polarization in SCI. In our study, we hypothesized that low-dose USW therapy combined with BMSC transplantation would be more effective than BMSC transplantation alone in improving nerve regeneration and functional recovery after SCI.

The success of cell transplantation depends on the ability to monitor the proliferation, differentiation, migration and distribution of implanted cells *in vivo*. Super-Paramagnetic Iron Oxide Nanoparticles (SPIONs) are an ideal magnetic resonance contrast medium with good biocompatibility and offer the possibility for non-invasive labeling and tracking of transplanted cells [17]. Stem cells transplanted in damaged areas for the purpose of tissue repair are a dynamic process. This requires SPIONs to maintain good proliferation and multi-directional differentiation potential of stem cells and not to interfere with the normal movement of stem cells and their ability to migrate [18]. In our study, we used SPIONs at a dose of 25 µg/ml to mark BMSCs and observed that BMSCs could be efficiently and safely labeled by SPIONs for long-term observation [19].
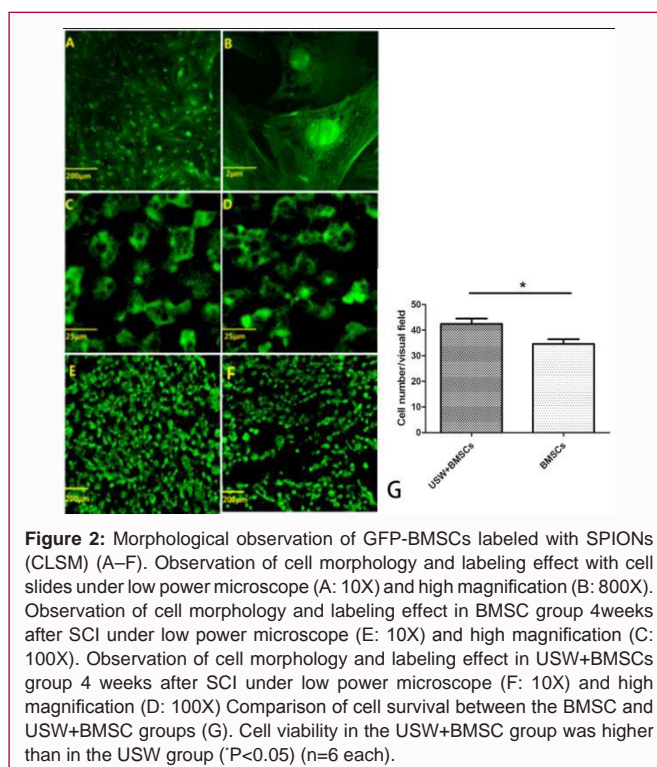
Materials and Methods

Materials

Female Sprague-Dawley rats aged 10 weeks, weighing 200 g to 220 g, were provided by the Experimental Animal Center of China
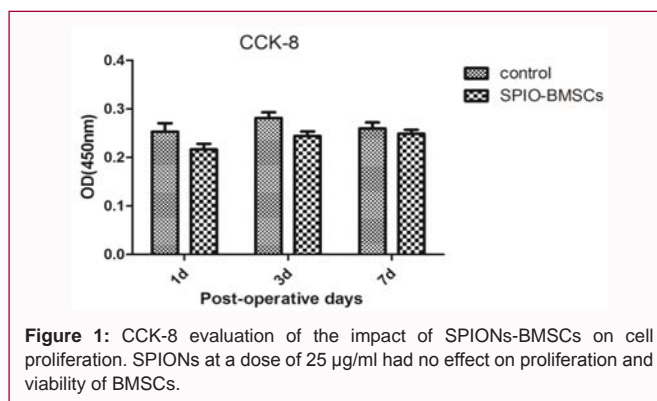
Figure 1: CCK-8 evaluation of the impact of SPIONs-BMSCs on cell proliferation. SPIONs at a dose of 25 µg/ml had no effect on proliferation and viability of BMSCs.
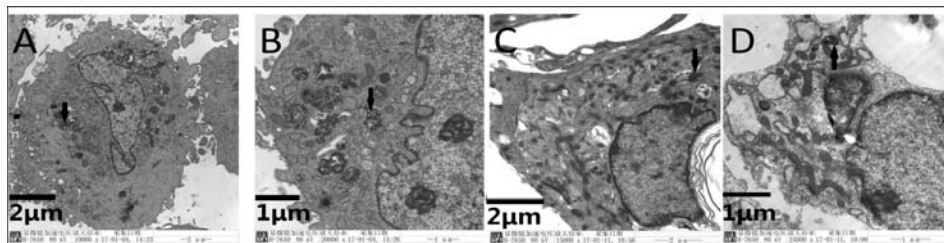

Figure 2: Morphological observation of GFP-BMSCs labeled with SPIONs (CLSM) (A–F). Observation of cell morphology and labeling effect with cell slides under low power microscope (A: 10X) and high magnification (B: 800X). Observation of cell morphology and labeling effect in BMSC group 4weeks after SCI under low power microscope (E: 10X) and high magnification (C: 100X). Observation of cell morphology and labeling effect in USW+BMSCs group 4 weeks after SCI under low power microscope (F: 10X) and high magnification (D: 100X) Comparison of cell survival between the BMSC and USW+BMSC groups (G). Cell viability in the USW+BMSC group was higher than in the USW group (*P<0.05) (n=6 each).

Medical University, Shenyang, China. Green Fluorescent Protein (GFP) Sprague-Dawley rats aged 4 weeks, weighing 60 g to 80 g, was provided by Mingsheng Company of Shanghai, China. The USW device (40.68 MHz, 40W; Shanghai Electrical Device Company) was controlled at about 11.58W for the first channel power. SPIONs (1 mg/ml; NANOEAST, China) were prepared with iron ion mass concentration of 25 µg/ml. A confocal laser scanning microscope (Nikon, Japan) and transmission electron microscope (TEM; TECNAI G2; FEI, Netherlands) were provided by China Medical University. All animal procedures were approved by Animal Ethics Committee of Shengjing Hospital of China Medical University. Animal care and surgical interventions were undertaken in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals.

Cell culture and labeling with SPIONs

Isolation and expansion of GFP-BMSCs was performed by attachment culture. The femur bones of GFP rats were excised and BMSCs were rinshed and dispersed into DMEM/F-12 (Gibco, USA) culture medium, containing 10% fetal bovine serum (Biological Industries, Israel), penicillin and streptomycin. Stem cells were

Figure 3: Morphological observation of GFP-BMSCs labeled with SPIONs (TEM). Black arrow represents SPIONs. The mitochondria and lysosomes had crumb or dot particles (A,B). At 4 weeks post-SCI, BMSCs survived and had irregular polymorphic forms. SPIONs were visible in the cytoplasm in the BMSC and USW+BMSC groups (C,D) (n=6 each).

incubated in a humidified atmosphere at 37°C with 5% CO_2, and sub-cultured when they reached 80% to 90% confluence.

We diluted the concentration of SPINOs from 1 mg/ml into 25 μg/ml with DMEM/F-12, and then the culture medium was added to the third generation of BMSCs and incubated for 48 h. The cells were washed three times with PBS to remove iron ions that did not enter the cell. The cells were digested with 0.25% trypsin. We diluted the digested cells with DMEM/F-12 culture medium to 1×10^6/μl for transplantation. Cell aggregates were obtained by ultracentrifugation at 1200 r/min for 5 min and fixed in 2% glutaraldehyde and 2% paraformaldehyde for TEM observation. Another small number of cells were operated the labeling effects with Prussian blue staining.

Prussian blue staining for iron assessment

We fixed the cell slides with 40 g/L paraformaldehyde for 30 min with washed three times with Phosphate buffered saline (PBS: HyClone, USA). We next added a mixture of 500 μL 2% ferrouspotassium cyanide solution and 500 μL 6% hydrochloric acid solution on the cell slide and incubated it at the temperature of 37°C for 30 min, and washed three times with PBS. We counterstained the cells with 1% Nuclear Fast Red solution for 30 s and washed three times with PBS. Finally, we calculated the number of labeled cells and labeling rate using a microscope (Olympus, Japan).

Cell Counting Kit-8 (CCK-8) method to detect cell proliferation

We digested the third generation of BMSCs and SPIONs-BMSCs (marked 1, 3 and 7 days, respectively) with 0.25% trypsin and diluted the digested cells to 5×10^4/ml. We used 96-well plates to cultivate the cells and each group had six plates with 100 μL cell suspension. The blank control group consisted of 100 μL cell culture medium. The 96-well plates were incubated in a humidified atmosphere at 37°C with 5% CO_2 for 8 h, and then we added 10 μL cholecystokinin into each plate. We incubated the 96-well plates for 2 h, and the absorbance at 450 nm was determined by Microplate Reader (Bio Tek, USA).

Animal groups and procedures for inducing SCI

The rats (200 g to 220 g) were anesthetized with 10% chloral hydrate (0.33 mg/100 ml), then the thoracic back area was shaved and disinfected with 75% alcohol, and laminectomy with the duramater intact was performed at the T9-T11 spinal cord level. Contused incomplete SCI was induced by dropping a 9 g weight rod from a 10 mm height onto the exposed dorsal surface of the spinal cord using an Allen's impactor [20]. During the recovery period, the rats were maintained at 37°C in a heating chamber. The postoperative care included bladder expression (once or twice daily until bladder function recovered) and prophylactic kanamycin (1 mg/kg) was regularly injected for 3 days after surgery.

One hundred and twenty female Sprague-Dawley rats were randomly divided into five groups of 24: (1) sham-operated group: rats only received laminectomy without SCI; (2) control group:rats were injected with 5 μL DMEM/F12 at the moment after SCI; (3) USW group: rats were treated with low-dose USW therapy 24 h after SCI, 7 min once daily until the rats were sacrificed; (4) BMSC group: 5 μL (10^6/μL) BMSCs were injected into the site of injury at the moment after SCI; and (5) USW+BMSC group: BMSCs were immediately injected into the injury site after SCI, and the rats were treated with USW therapy for 24 h after SCI until they were sacrificed. The Basso-Beattie-Bresnahan (BBB) scale test was performed before SCI and at 1, 3 and 7 days after. Expression of iNOS, Arg-1 and ED-1 in the spinal cord after SCI was tested by immunohistochemistry.

Twelve female Sprague-Dawley rats were randomly divided into USW+BMSC and BMSC groups for 4 weeks (n=6 in each group). In the BMSC group, 5 μL (10^6/μL) SPIONs-BMSCs were injected into the site of injury immediately after SCI; and in the USW+BMSC group, 5μL (10^6/μL) SPIONs-BMSCs were immediately injected into the injury site after SCI, and the rats received USW therapy 24 h after SCI, 7 min once daily, until the rats were killed. We counted the number of surviving SPIONs-BMSCs in the damaged area under Confocal Laser Scanning Microscope (CLSM) and observed the changes in morphology with TEM. Prussian blue staining was used for iron assessment and SPIONs-BMSCs distribution.

Behavioral analysis

We used a 21-point BBB scale to evaluate the hind limb motor function, and the tests were performed before the SCI operation and at 1, 3 and 7 days after SCI [21,22]. The rats were placed in an open field and allowed to move freely for 4 min, and this test was operated by two observers blinded to the groups.

Immunohistochemistry

The experimental animals were anesthetized with chloral hydrate (0.3 mL/100 g) and the excised spinal cord tissues were fixed with 4% (v/v) formaldehyde and embedded in paraffin. The tissues were sectioned at 3 μm thickness, washed and blocked with 10% fetal bovine serum for 20 min at 37°C, and incubated with primary antibodies against ED-1 (1:2000; Millipore, USA), iNOS (1:150; Bioss, China) and Arg-1 (1:200; Bioss), followed by incubation overnight at 4°C. The tissue slices were preserved with H_2O_2/methanol (Beyotime, China) to block endogenous peroxidase activity for ~20 min, and incubated with secondary antibody (goat anti-rabbit; Beyotime) at room temperature for 20 min. The signals were detected by staining with Diaminobenzidine, and the nuclei were counter-stained with hematoxylin. Tissues were examined under a microscope.
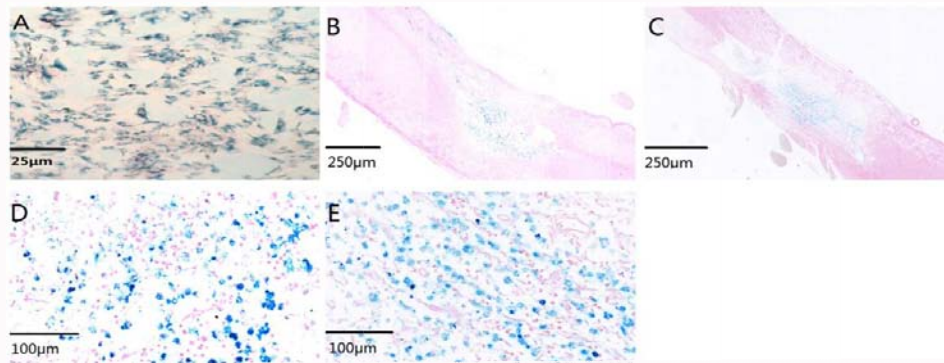
Figure 4: Prussian blue staining for iron assessment and cell distribution. The positive staining rate was ~98% (A). Distribution of BMSCs transplanted 4weeks after SCI in BMSC (B) and USW+BMSC (C) groups. Cells migrated to the rostral section and caudal end of the damaged area of spinal cord in the BMSC group, and gathered in the central area of the injured spinal cord in the USW+BMSC group.
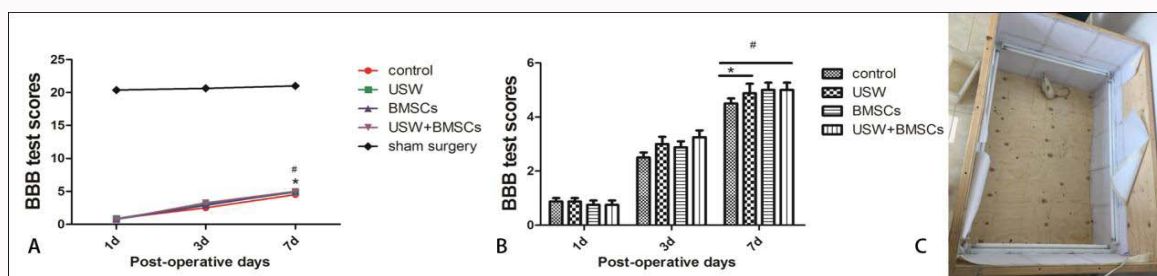
Figure 5: Effect of USW therapy combined with BMSC transplantation on motor performance in rats after SCI. BBB scale (C). At 7 days after SCI, the USW and USW+BMSC groups had better functional recovery compared with the control group (A,B) (USW *vs.* control: $^*P<0.05$; USW+BMSCs *vs.* control: $^\#P<0.05$, respectively) (n=8 each).

Statistical analysis

GraphPad Prism 5 was used for all statistical analyses. Statistical differences were evaluated by one-way analysis of variance followed by Bonferroni's multiple comparison tests. $P<0.05$ denoted statistical significance.

Results

CCK-8 detection of the proliferation of SPIONs-BMSCs

Labeling BMSCs with 25 µg/ml SPIONs made no difference to the proliferation capacity of stem cells after 1, 3 and 7 days (Figure1).

Morphological observation of GFP-BMSCs labeled with SPIONs and comparison of cell survival in different groups

CLSM showed green fluorescence in the nucleus and cytoplasm and a fluorometric rate of nearly 100%. Cells were long and fusiform (Figure 2A). At high magnification, SPIONs were crowded around the nucleus and cytoplasm (Figure 2B). At 4 weeks after SCI, BMSCs survived and had irregular polymorphic forms; SPIONs were also visible in the cytoplasm (Figure 2C and 2D). At 4 weeks after USW therapy, we observed a higher number of surviving cells in the USW+BMSC group (42.40 ± 6.535) (Figure 2E) compared with the BMSC group (34.60 ± 5.967) (Figure 2F and 2G) (P<0.05) (Figure 2).

TEM clearly showed the structure and morphology of the cells. The cytoplasmic mitochondria and lysosome had crumb or dot particles and no SPIONs in the nuclei. SPIONs were not uniform in size and the larger particles reached hundreds of nanometers (Figure 3A and 3B). At 4 weeks after SCI, BMSCs had morphological changes and SPIONs were visible in the cytoplasm (Figure 3C and 3D) (Figure 3).

### Prussian blue staining for iron assessment and cell distribution

Prussian blue staining of the spinal cord sections showed that BMSCs were long and spindle-shaped, and SPIONs were mostly distributed around the nucleus in the cytoplasm. We selected ten random microscopic fields to calculate the ratio of SPIONs labeled cells, and found that the positive rate was ~98% (Figure 4A). At 4 weeks after SCI, cells in the BMSC group migrated to the rostral section and caudal end of the damaged area of spinal cord (Figure 4B). Cells in the USW+BMSC group gathered in the central area of the injured spinal cord (Figure 4C). Figure 4D shows the number of cells with positive granules in the BMSC group and Figure 4E shows the number in the USW+BMSC group (Figure 4).

USW therapy combined with BMSC transplantation promotes functional recovery after SCI

We used the BBB scale to evaluate the effects of USW therapy combined with BMSCs on functional recovery. The rats after SCI were all in the paralysis of lower limbs, and BBB score was 0-2 points the day after surgery. There was no influence on motor function in the sham-operated group after SCI; at 1 and 3 days after SCI each model showed different degrees of recovery in movement, although the difference was not significant. At 7 days after SCI, the USW and USW+BMSC groups had better motor recovery than the control group had (USW *vs.* control P<0.05; USW+BMSC *vs.* control P<0.05) (Figure 5A-5C).

USW therapy combined with BMSC transplantation reduces early inflammation after SCI

ED-1 is a single-chain glycoprotein that specifically binds to the lysosomal membranes of activated macrophages, which can detect
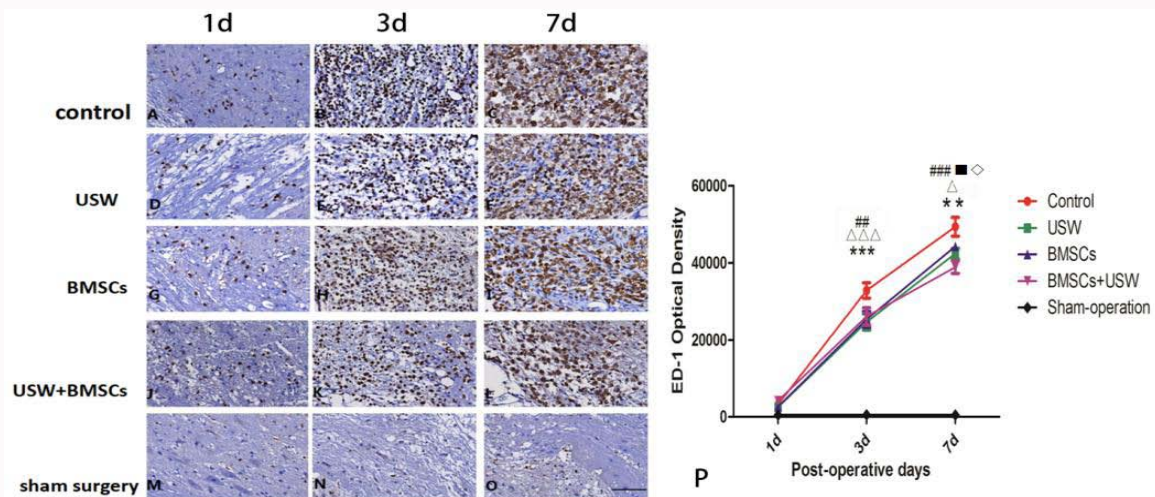
Figure 6: ED-1 expression at 1 to 7 days after SCI (A–O) and data analysis of all groups with times after surgery (P). At 3 days after SCI, ED-1 expression was lower in the USW, BMSC and USW+BMSC groups than in the control group (USW vs. control: ***P<0.001; BMSC vs. control: ***P<0.001; USW+BMSC vs. control: ##P<0.01, respectively) (B,E,H,K). After 7 days, ED-1 expression was lower in the USW, BMSC and USW+BMSC groups than in the control group (USW vs. control: **P<0.01; BMSC vs. control: *P<0.05; USW+BMSC vs. control: ###P<0.001) (C,F,I,L). At 7 days after SCI ED-1 expression decreased in the USW+BMSC group compared with the USW and BMSC groups (USW+BMSC vs. USW: ▪P<0.05; USW+BMSC vs. BMSC: *P<0.05) (I,L) (n=8 each).
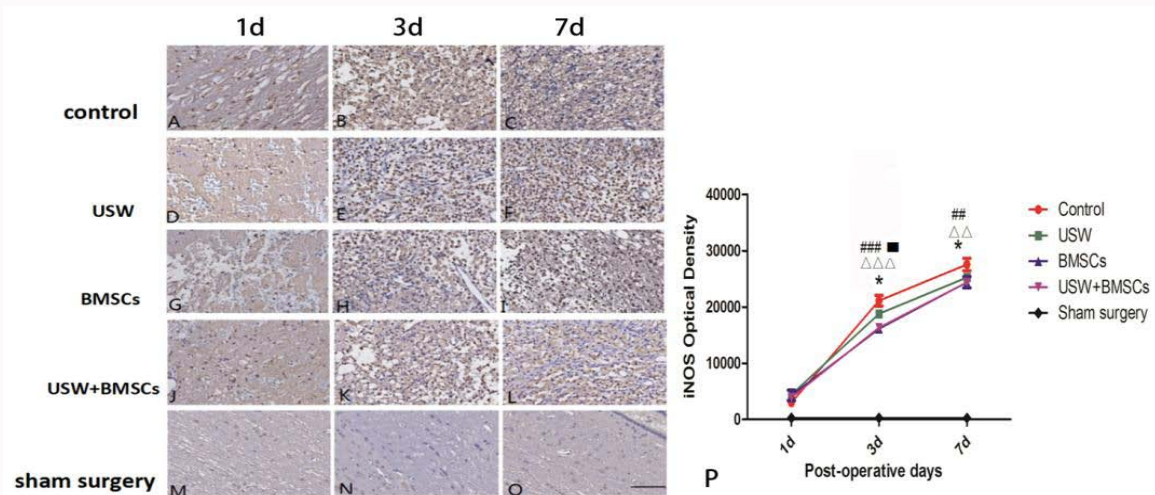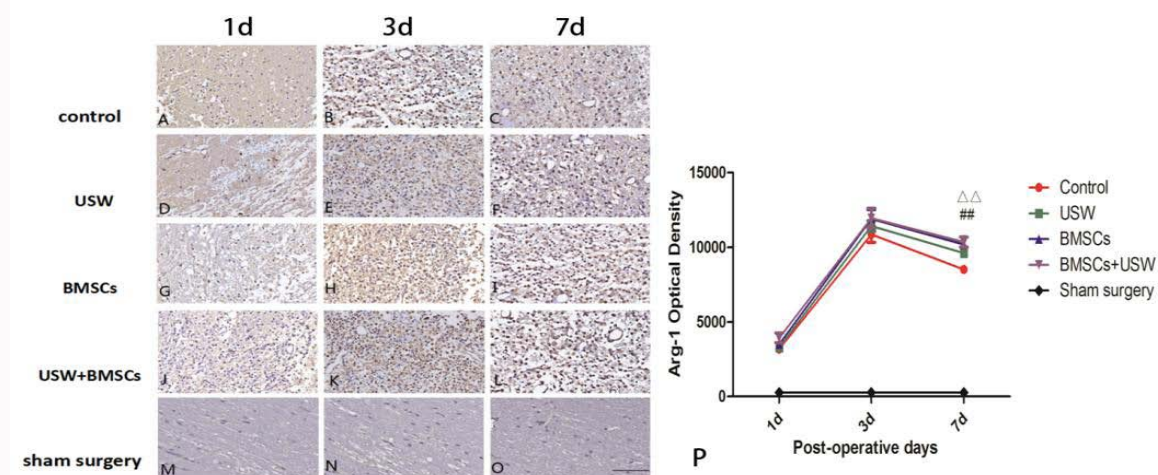

Figure 7: iNOS expression at 1 to 7 days after SCI (A–O) and data analysis of all groups with times after surgery (P). Expression of iNOS was decreased in the USW, BMSC and USW+BMSC groups compared with the control group at 3 days after SCI (USW vs. control: ·P<0.05; BMSCs vs. control: ***P<0.001; USW+BMSCs vs. control: ###P<0.001) (B,E,H,K). Expression of iNOS was higher in the USW than USW+BMSC group (USW+BMSC vs. USW: ▪P<0.05) (E,H,K) at 7 days after SCI. Expression of iNOS was lower in the USW, BMSC and the USW+BMSC groups compared with the control group (USW vs. control: ·P<0.05; BMSC vs. control: **P<0.01; USW+BMSC vs. control: ##P<0.01) (C, F, I, L) (n=8 each).

active macrophages after SCI [23]. ED-1 is mainly expressed around damaged areas with a fragmented or foamy distribution (Figure 6). The sham-operated group had few ED-1-positive cells (Figure 6M-6O). Expression of ED-1 increased significantly in the experimental groups compared with the sham-operated group, especially 7 days after SCI. At 3 days after SCI, ED-1 expression in the USW, BMSC and USW+BMSC groups was less than in the control group (Figures 6B,6E,6H,6K). At 7 days after SCI, there were significantly fewer E-1-positive cells in the USW, BMSC and USW+BMSC groups than in the control group (Figures 6C,6F,6I,6L). At 7 days after surgery ED-1 expression was decreased in the USW+BMSC group compared with the USW and BMSC groups (Figures 6I,6L). These data indicated that the early inflammatory responses were alleviated by combination of USW therapy and BMSC transplantation (Figure 6P).

USW therapy combined with BMSC transplantation promotes polarization of macrophages after SCI

We used optical density to analyze the polarization markers iNOS (M1) (Figure 7) and Arg-1(M2) (Figure 8). iNOS was mainly expressed in the cytoplasm of neurons, astrocytes and microglia, and its expression was significantly increased in each experimental group compared with the sham-operated group at 3 and 7 days after SCI (Figure 7P). M2 macrophage polarization marker Arg-1was expressed in the cytoplasm of cells in the gray and white matter of spinal cord tissue. Arg-1 expression was increased and reached a peak at 3 days after SCI (Figure 8P). After USW therapy and BMSC transplantation, expression of iNOS followed the same trend as ED-1 expression, and was significantly decreased in the USW, BMSC and USW+BMSC groups compared with the control group at 3 and 7 days after SCI. In

Figure 8: Arg-1 expression at 1 to 7 days after SCI (A-O) and data analysis of all groups with times after surgery (P). At 7 days after SCI, Arg-1 expression was higher in the BMSC and USW+BMSC groups than in the control group (¨P<0.01, **P<0.01) (F,I,L) (n=8 each).

contrast, expression of Arg-1 in the BMSC and USW+BMSC groups was higher than in the control group at 7 days after SCI. Expression of iNOS was lower in the BMSC and USW+BMSC groups than that in the USW group at 3 days after SCI, and Arg-1expression was higher in the BMSC and USW+BMSC groups than in the control group. Low-dose USW therapy combined with BMSC transplantation alleviated early inflammation (ED-1) through reduced M1 macrophage activation, and polarization of M2 macrophages, and BMSC transplantation had the dominant effect. These results indicated that M1 macrophage polarization could be reversed toward the M2 phenotype using USW therapy combined with BMSC transplantation.

Discussion

Polarization of macrophages plays a critical role in inflammation during SCI [24]. Macrophage transformation from a proinflammatory (M1) subtype in the early stages after SCI to a pro-healing (M2) phenotype in later stages is associated with microenvironmental changes [25]. Accumulating evidence supports that M1 macrophages can be induced by interferon-γ or lipopolysaccharide and secrete proinflammatory cytokines, chemokines, reactive oxygen as well as participate in immune response and surveillance process. M2 macrophages can be induced by Interleukin (IL)-4, IL-10, and transforming growth factor-β. M2 macrophages have weak antigen presentation and play a critical role in down regulating the immune response by secreting inhibitory cytokines [26-28]. Polarization to the M1 phenotype is induced rapidly after injury and persists for a long time, but polarization to the M2 phenotype is transient, which is consistent with our findings [29]. ED-1 and iNOS expression increased and reached a peak at 7 days after SCI and was sustained for a long time, whereas Arg-1 reached a peak at 3 days after SCI and lasted only a few days. Phenotypic polarization to the M2 phenotype is a novel strategy to alleviate early impairment following SCI.

We studied the synergistic effect of low-dose USW therapy and BMSC transplantation in a rat model of SCI. Combination therapy changed the immune microenvironment of injury sites by decreasing expression of M1 macrophages (iNOS) and increasing the number of M2 macrophages (Arg-1). More importantly, expression of ED-1 and iNOS in the USW, BMSC and USW+BMSC groups was lower than in the control group at 3 and 7 days after SCI, whereas Arg-1

expression in the BMSC and USW+BMCS groups decreased, more slowly than in the control group after they peaked at 3 days after SCI. We also observed that expression of iNOS was lower in the BMSC and USW+BMSC groups than in the USW group at 3 days after SCI, and Arg-1 expression was higher in the BMSC and USW+BMSC groups than that in the control group. Expression of iNOS in the BMSC and USW+BMSC groups was lower than in the USW group at 3 days after SCI. Arg-1 expression was higher in the BMSC and USW+BMSC groups than in the control group. We demonstrated that combination therapy had a positive effect on promoting polarization of macrophages from M1 to M2 phenotype, and reduced inflammation in the early stages after SCI. In the early stages, transplanted BMSCs may play a greater role in inducing macrophage polarization from M1 to M2 phenotype, which agrees with the conclusion that BMSC transplantation favored the development of M2 macrophages, while preventing the development of M1 macrophages after SCI. BMSCs promote secretion of ectodomain of sialic acid-binding Ig-like lectin-9 and monocyte chemoattractant protein-1, which are inducers of M2 macrophages and help to modulate polarization from M1 to M2 phenotype [30]. Improvement of motor function was demonstrated by higher BBB scale score in the USW and USW+BMSC groups at 7 days after SCI; therefore, we concluded that USW therapy makes a contribution to functional recovery, which is in accordance with our previous study [12]. The possible mechanism is that USW therapy improves the microenvironment by promoting blood circulation and nutrition metabolism. This alleviates inflammation and reduces tissue edema, which provides a cellular microenvironment that favors stem cell survival and plays a role in damage repair, functional recovery and nerve reconstruction. This means that SCI rats with USW therapy have better motor recovery and survival of BMSCs than those without USW therapy, as we observed through CLSM.

The main purpose of stem cell transplantation is to reconstruct damaged tissue with functional cells. We injected BMSCs into the lesion site with a microsyringe, which is a less invasive way and likely to deliver the cells into the cerebrospinal fluid. Transplanted BMSCs exert selective anti-inflammatory effects, alleviate harmful inflammatory responses, and demonstrate that stem cells have neuroprotective and regenerative potential in the impaired nervous system [31,32]. We also observed that BMSCs play a vital role in

promoting M1 macrophage polarization into the M2 phenotype, and M2 macrophage polarization contributes to the ability of BMSCs to ameliorate SCI [33]. The possible mechanism is that M1 macrophages promote differentiation of BMSCs into astrocytes, while M2 macrophages promote differentiation of BMSCs into neurons and oligodendrocytes. The differentiated neurons can integrate with the host neurons, thus playing a critical role in promoting nerve regrowth and functional recovery after SCI. In other words, M2 macrophages exude high levels of growth factors and cytokines (such as brain-derived neurotrophic factor, vascular endothelial growth factor and basic fibroblast growth factor) compared with M1 macrophages. These factors can provide good living conditions for BMSCs, give them nourishment *in situ*, and make them more inclined to differentiate into neurons [34]. In the present study, Prussian blue staining showed that cell in the BMSC group migrated to the rostral section and caudal end of the damaged area of spinal cord, and the cells in USW+BMSC group gathered in the central area. It has been shown that stromal cell-derived factor-1 [or chemokine CXC ligand (CXCL)12, expressed by the dorsal corticospinal tract and meninges] and its receptor, CXCR4 (expressed by ependymal cells around the central canal) may be involved in regulation of transplanted cell migration [34]. Injected neural stem/progenitor cells are prone to migrate to areas of high CXCL12 expression, and M2 macrophages can promote neuronal differentiation and restrain the movement of transplanted stem cells to the injection area [35]. Therefore, the cells play a role in replacing lost cells and enhancing the interactions of engrafted cells with host neurons. USW therapy improves the microenvironment by reducing inflammation and tissue edema, thereby preventing ischemic anoxic necrosis of tissue cells around the site of injury and providing an environment that favors BMSC survival and function [13].

In vivo cell-tracking methods allow monitoring of the survival, early localization, migration, and differentiation of transplanted cells in real time. Direct labeling and indirect marking are two methods of cell labeling. In direct labeling, radiotracers, nanoparticles and quantum dots can be used to label the cells before transplantation without genetic modification [36-38]. In indirect labeling, target cells can be genetically modified by the introduction of a reporter gene that can monitor stem cell behavior longitudinally and precisely [39]. SPIONs are biodegradable and biocompatible, which means they can enter the plasma-iron pool and combine with hemoglobin, or participate in other metabolic processes after being metabolized by the cell. When they are consumed by other tissue cells or macrophages, false-positive reactions occur [40]. We used SPIONs to track the transplanted BMSCs after SCI through CLSM, TEM and Prussian blue staining for long-term dynamic functional assessment [41]. In summary, SPIONs can be used as a noninvasive tracer safely and effectively in animal models.

Conclusion

Our results indicate that USW therapy combined with BMSC transplantation can improve functional recovery through reducing macrophage activation and inflammation. Combination therapy actively promotes polarization of macrophages from M1 to M2 phenotype, and BMSC transplantation plays the dominant part. USW therapy improves the microenvironment that favors stem cell survival and plays a role in damage repair. The polarization state of macrophages influences the distribution and migration of BMSCs. BMSCs can be efficiently and safely labeled by SPIONs and used for

long-term observation.

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