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Research Article

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Gene Therapy of Spinal Cord Injury Using Gene-Modified Bone Marrow Stromal Cells With Fibromodulin Expressing Adenoviral Vector in A Rat SCI Model

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Abstract

Background: *Spinal cord injury (SCI) can lead to various pathological changes which create an inappropriate environment for repair. The most important of such changes are glial scar and inhibition of neuronal growth in the injured site. Exogenous administration of genes that enhance neuronal survival, synaptic plasticity, and neurotransmission has been considered as a potential approach for treating SCI. Fibromodulin is one of those genes which can decrease TGF-β1 and increase MMP-2 expression, and consequently leads to a reduction in the glial scar, promotes the growth of axons, macrophage activation, and elimination of physical and molecular barriers of neuronal growth that will end with improvement in motor performance. Moreover, bone marrow stromal cells (BMSCs) can be a promising therapeutic strategy for SCI because they can secrete neural growth factors as well as differentiate into neurons.*

Methods: *We randomly divided rats into four groups, each consisting of thirteen rats. The first group was administered normal saline, the second group received BMSCs, the third group received BMSCs infected with a beta-galactosidase expressing adenoviral vector, and the fourth group received BMSCs infected with a Fibromodulin expressing adenoviral vector. After inducing spinal cord injury using the weight-dropping method under general anesthesia, BMSCs were injected on the fourth-day post-injury. A Basso, Beattie, and Bresnahan (BBB) score test was conducted for six weeks. At the end of the fourth week, biotin dextran amine (BDA) was intracerebrally injected, and tissue staining was carried out two weeks after the injection.*

Results: *The BBB locomotor score test was applied for six weeks. There were significant differences in BBB locomotor scale between the first and the fourth groups. The mean score of the first group in the sixth week was 5.60, while it was 9.60 for the fourth group. There were significant differences in axon counting between the groups ($P<0.000$). The average number of axons counted from the first to the fourth group was 87.07, 466.33, 474.13, and 829.40, respectively.*

Conclusions: *Consequently, our results highlight the therapeutic potential of the Fibromodulin expressing BMSCs for treating SCI.*

Keywords: Fibromodulin, Bone marrow Stromal Cells, Gene Therapy, Cell Therapy, Spinal Cord Injury.

Introduction

Spinal cord injury is a prevalent disease worldwide. According to a 2013 report by the World Health Organization (WHO), the exact global estimate of spinal cord injury prevalence is unknown, but it occurs approximately in 40 to 80 cases per million populations [1]. Every year, between 250,000 and 500,000 patients suffer from spinal cord injury globally [2]. In the United States, the most recent estimate of the annual incidence of spinal cord injury (SCI) is about 54 cases per one million people. This means about 18,000 new SCI cases occur each year, and approximately 282,000 individuals are estimated to be living with SCI [3]. However, it is estimated to be between 318.45 to 440 per million people in Iran [4, 5]. Spinal cord injury can cause paraplegia, which is the loss of muscle function in the lower half of the body, or quadriplegia, which is the partial or total loss of function in all four limbs. This can devastate a patient's quality of life, life expectancy, and economic burden [6]. While there have been improvements in quality of life, spinal cord injury remains the leading cause of disability and mortality. Unfortunately, there is currently no fully restorative therapy for SCI, so prevention is key. After spinal cord injury, many pathological events occur, including glial scar formation and inhibition of neuronal growth at the lesion site. Therefore, new therapeutic approaches should be considered that focus on inhibiting glial scar formation and promoting neuronal growth [7, 8]. Studies have shown that bone marrow stromal cells (BMSCs) transplantation at the lesion site can play a crucial role in the treatment of spinal cord injury [9-13]. BMSCs are adult stem cells that originate from bone marrow. One of the clinical benefits of BMSC transplantation therapy is that bone marrow harvesting is more convenient, and there is no immunological issue because the stem cells can be collected from the patients themselves [14]. BMSC cells also have the potential to differentiate into astrocytes and neurons, and release neurotrophic factors that stimulate axonal growth and promote neuronal survival [15, 16]. Therefore, they are considered a promising cellular source for clinical use in autologous grafts for neurological disorders [17-20]. Fibromodulin (FMOD) is a Keratan sulfate proteoglycan expressed in skin, cornea, and sclera and connective tissues such as cartilage, ligaments, tendons, and dermal tissues. Fibromodulin (FMOD) is a Keratan sulfate proteoglycan expressed in skin, cornea, sclera, cartilage, ligaments, tendons, and dermal tissues. FMOD is a member of the small leucine-rich proteoglycans (SLRPs) family and glycoproteins, including Lumican, Decorin, and Biglycan [21-24]. FMOD is upregulated in fibrotic, inflammatory, and wound-healing processes in the lung, kidney, liver, and skin, and it is a modulator of TGF-β1 activity [22-26]. In addition, studies have shown that overexpression of Fibromodulin leads to a reduction in TGF-β1 and an induction in MMP-2 secretion, resulting in a decrease in glial scar formation, an increase in axonal growth, macrophage activation, and the removal of all physical molecular barriers to neural growth. This ultimately leads to an improvement in motor activity [27-29]. The commonly used viral vectors for gene therapy of SCI are adenovirus, AAV, and lentivirus. AAV and lentivirus lead to the constant expression of the transgene, whereas adenoviral vectors result in transient expression. Since this study is not interested in long-term Fibromodulin expression, adenovirus would be more appropriate for the transient expression of Fibromodulin. Additionally, adenoviral vectors have many advantages, such as high transduction efficiency in quiescent and dividing cell types, high levels of interesting gene expression in the host, and the ability to produce high titers [30-36]. In this study, we engineered BMSCs to secrete Fibromodulin using an ex vivo adenoviral vector (AdV) transduction technique. These modified BMSCs were transplanted into the injured rat spinal cord, and corticospinal tract (CST) projections were visualized using biotin dextran amine (BDA). Behavioral analysis was performed over a six weeks period using open-field locomotion.

Material and Methods

Animals

For all experiments, male Sprague-Dawley (SD) rats aged between six to eight weeks and weighing between 210 to 230 grams were used. These rats were obtained from the Razi Vaccine and Serum Research Institute in Iran. All experimental protocols were undertaken in compliance with the Institutional Animal Care & Use Committee (IACUC) standards and approved by the Zanjan University of Medical Sciences Ethics Review Board (IR.ZUMS.REC.1399. 881).After spinal cord contusion, all rats received antibiotics treatment (cefazolin 50 mg/kg) and underwent urinary bladder massage two or more times a day until they recovered their ability to urinate spontaneously.

Isolation of Rat Bone Marrow Stromal Cells (BMSCs) and Characterization

Bone marrow stromal cells from rats were isolated following a previously described protocol [37]. The tibia and femur bones were removed, cleaned, and extracted from rats. The leg's skin, fur, and muscles were peeled off, and the bones were rinsed in 70% ethanol for one minute before being placed in a sterile PBS-filled petri dish. After this stage, all procedures were performed in a biological safety cabinet. The bone ends were cut with scissors, and the bone marrow was flushed into a 50ml tube with sterile PBS using a 22G needle attached to a 5ml syringe. This step was repeated two to three times for each bone. The cell suspension was passed through a 70 µm cell strainer to remove bone debris and blood aggregates. The isolated BMSCs were characterized according to a previously established protocol [38].

Cell Culture

To prepare the bone marrow cells for culture, 10 ml of fresh DMEM medium (with 10% FBS and 1% pen-strep) was added to the extracted cells. The cells were then seeded in a 25cm2 flask and incubated in a 37°C and 5% CO2 incubator for 3 hours. After 3 hours, the medium was replaced with a fresh DMEM medium. As the BMSCs can adhere to the surface of the culture flask, the suspension cells were excluded from this step. The culture medium was replaced with fresh medium every day for the first three days and then every 3 or 4 days. After approximately 2-3 weeks, the cells had reached efficient confluences and could be subcultured when they reached a confluence of ≥60%. The culture medium was removed, and the monolayer cells were rinsed with PBS. 2ml Trypsin-EDTA was added to the washed cells and incubated for 2-5 minutes at 37°C until the cells were detached. To inhibit trypsin action, 5-10 ml media containing serum were added. The cells were collected in a tube and centrifuged for approximately 5 minutes at 400g. Then, the cell pellet was resuspended in fresh medium and dispensed into two flasks.

Adenoviral Vector

For this study, replication-defective adenoviruses containing bovine Fibromodulin (Ad5-FMOD), and adenoviruses containing the β-galactosidase gene (Ad5-LacZ) were kindly gifted by Dr. Paul Kingstone (The University of Manchester, U.K) [39].

BMSCs Transduction by Ad5-FMOD Vector

To determine the Adenoviral titration, a plaque assay was conducted in HEK 293 cells. Two days before transduction, BMSC cells were plated in a 24-well plate with 2 × 105 cells per well. The Ad5-FMOD and Ad5-LacZ were diluted in RPMI 1640 serum-free culture media (Gibco, Invitrogen) and added to the cells with a multiplicity of infection (MOI) of 100 pfu/cell. The cells were then incubated at 37°C. Four hours after transduction, the media was removed, and the cells were washed with PBS and cultured again in fresh medium with 15% FBS for 48h.

Evaluation of Fibromodulin Expression by RT-PCR

The Qiagen RNeasy Mini Kit (Cat.No: 74104) was used for extracting total RNA from transduced cells, following the manufacturer's instructions. A total of 1 µg of RNA was utilized to generate cDNA, using the Qiagen One-Step RT-PCR cDNA Synthesis Kit as per the manufacturer's specifications. The RT-PCR was carried out with the following primers:
FMOD forward primer (FMF1) 5′-TGAAGGCAGCACCTGACCGC-3′, FMOD reverse primer (FMR1) 5′-ACGCCTTGGCTTCTCCTGCC-3′ (189bp). β-Actin forward primer 5′ AAGCAGGAGTATGACGAGTC-3′, β-Actin reverse primer 5′ CCGTTCCAGTTTTTAAATCC-3′ (207bp).

The PCR was performed for 40 cycles under the following conditions: 30 min at 50°C, 15 min at 95°C, 45 sec at 94°C, 45 sec at 63°C, 1 min at 72°C.

Spinal Cord Injury Rat Model

To perform spinal cord injury, a "weight dropping" method was utilized. Thirteen rats were randomly assigned to each of the four groups. All rats were anesthetized by a single dose of intraperitoneal injection of 87 mg of ketamine per kg of body weight and 10 mg of xylazine per kg of body weight. To prevent dryness of the cornea, a drop of mineral oil was used. The thoracic area was shaved and disinfected with povidone-iodine solution. Then, under a microscope, laminectomy surgery was performed on the tenth thoracic vertebra (T10). The spinal cord was injured by dropping a 10-gram metal rod from a 50-mm distance onto the exposed spinal cord (T10 vertebra). The wound was closed using chromic catgut (4/0) for the muscle and nylon suture (3/0) for the skin. After the surgery, rats were given Cefazolin (50 mg/kg BW/day intramuscular) for three days and placed on a 37°C heating blanket overnight [40-42].

Bone Marrow Stromal Cells Transplantation (BMSCT)

Four days following a spinal cord contusion, 2x105 transduced cells were suspended in phosphate-buffered saline (PBS). Rats were then fixed in a stereotaxic device, and the cells were injected using a 5µl Hamilton syringe to a depth of 1.5mm in the caudal border of the lesion site for 120 seconds. The first group of rats received normal saline, the second group was administered with BMSCs, the third group was given BMSCs infected with adenovirus expressing beta-galactosidase, and the fourth group received BMSCs infected with adenovirus expressing Fibromodulin.

Behavioral Analysis

The behavioral functions of the animals were evaluated one week after cell injection. The locomotor BBB test was used to assess their progress for six weeks after surgery. The BBB test is an open field score that ranges from complete hind limb paralysis (Zero) to normal movement (Twenty-one). Two observers, who were unaware of the treatment, scored the animals according to the BBB scale [43-44].

BDA Anterograde CST Tracing

To trace the corticospinal tract (CST), anterograde tracing was performed using BDA, which was administered as per standard procedures. The experiment involved 12 rats, with three rats randomly selected from each group. The rats were anesthetized, and their skulls were secured using a stereotaxic device. The surgical area was cleansed and sanitized with an iodine swab. A craniotomy was then performed, creating a hole approximately 1.0 mm in diameter and depth, at a location 2 mm lateral and 1.6 mm caudal to the bregma. Using a 5µl Hamilton syringe, a slow injection of 1 µl of 10% BDA (Life Technologies, Cat N: D-1956) was administered into the cerebral motor cortex at a depth of 1.5 mm for 160 seconds. The BDA was injected two weeks before the rats were sacrificed [45].

Histological Procedures

To prepare for analysis, one centimeter of the spinal cord with the lesion at the midpoint was cut and embedded in paraffin. The embedded spinal cords were cut into serial transverse sections, each 5-µm thick with a 200 µm interval, using a freezing microtome (Rotary Microtome, YD-2508). BDA labeling was performed as previously described. Briefly, the sections were rinsed in 0.1M Tris-buffered saline (TBS; pH 7.4) or PBS and treated with 0.6% hydrogen peroxide in TBS or PBS for 30 minutes to inhibit endogenous peroxidase activity. They were then incubated with avidin-biotin-peroxidase complex (VECTASTAIN® Elite® ABC HRP Kit; PK-6100, USA). After washing the sections, they were treated in diaminobenzidine tetrahydrochloride (DAB) and nickel chloride until the production of a dark reaction. Sections were photographed under a Nikon microscope (×40). The extent of the DAB labeled fibers in each section was quantified in a blinded manner using Scion Image software.

Statistical Analysis

Data were analyzed using SPSS v16 (Chicago, Inc., USA) and expressed as mean ± S.E.M. Statistical analysis involved one-way and two-way ANOVA, followed by post-hoc analysis with the Tukey test.

Results

Transferred Gene Expression in Vitro

To assess the expression level of Fibromodulin mRNA in vitro, reverse transcription-PCR (RT-PCR) was performed. The results showed that Fibromodulin mRNA expression was confirmed in the fourth group, as compared to the control groups (as shown in Fig. 1). The biological activity of an Adenoviral vector carrying the Fibromodulin gene was confirmed via a bioassay of TGF-β activity, as previously reported by P Ranjzad et al.

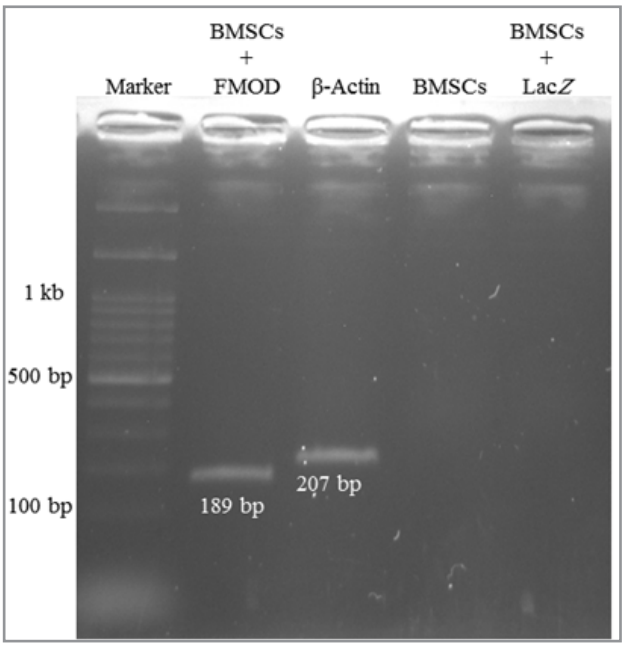

Figure 1: Identification of the Expression of Fibromodulin in BMSCs: RT-PCR of Fibromodulin mRNA of cells infected by Ad5-FMOD. There is a band of 189 bp on the lane of BMSCs+FMOD, and a band of 207bp for β-actin. Marker: 100 bp.

Recovery of Hind Limb Function

The behavioral analysis started one week after injecting cells and continued weekly for 6 weeks after spinal cord injury (see Fig. 2). To determine the locomotor recovery of rats, the BBB locomotion score was used, which considers the early (BBB score from 0 to 7), intermediate (8-13) and late phases (14-21) of recovery. According to statistical analysis, there were significant differences between groups during the 3rd, 5th, and 6th weeks. The probability values (p-values) were p=0.002, p=0.047, and p=0.006, respectively. However, no differences in BBB scores were found between all groups at other time points ($p<0.05$). No significant differences were observed until 1 week after injury between groups (p-value=0.325). In the third week, the first signs of locomotor function recovery were observed. There were significant differences between the fourth and control groups (p-value=0.00). At week six, the average score for the control group (group 1) was 5.60 ±1.140, for the second group was 8.00, for the third group was 8.50 ± 0.707, and for the fourth group was 9.60 ± 2.675. The average BBB score for the fourth group was significantly higher than the control group (p-value=0.03) over six weeks, indicating that the Fibromodulin gene had a significant effect on functional recovery.

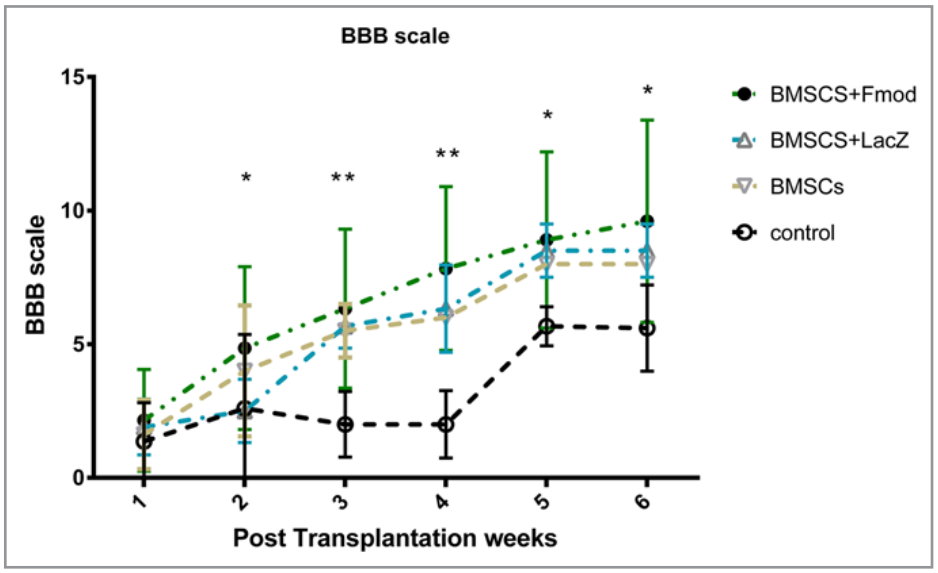

Figure 2: Functional Recovery: Open field locomotor evaluation using BBB scale was recorded every week after transplantation. Statistical analysis indicates that BBB scales in the BMSCs+fibromodulin transplantation group are significantly higher than those in the control group ($P < 0.05$).Values represent mean ± S.E.M. * $P \leq 0.05$ compared with the control group. S.E.M. standard error of the mean.

Quantification of CST Axons

Data collected from BDA anterograde tracing of CST fibers were analyzed to examine the diffusion pattern of sprouting CST fibers after spinal cord injury. The lowest axon count was obtained in the control group, with an average of 87.07 ± 46.75. The average number of axons was 466.33 ± 146.959 in group 2,

474.13 ± 109.149 in group 3, and 829.40 ± 139.006 in group 4. Groups 2, 3, and 4 showed significantly higher numbers of axons compared to the control group (P≤0.001). The number of axons in group 4 was statistically significant versus groups 2 and 3 (P ≤ 0.001) (Fig. 3).

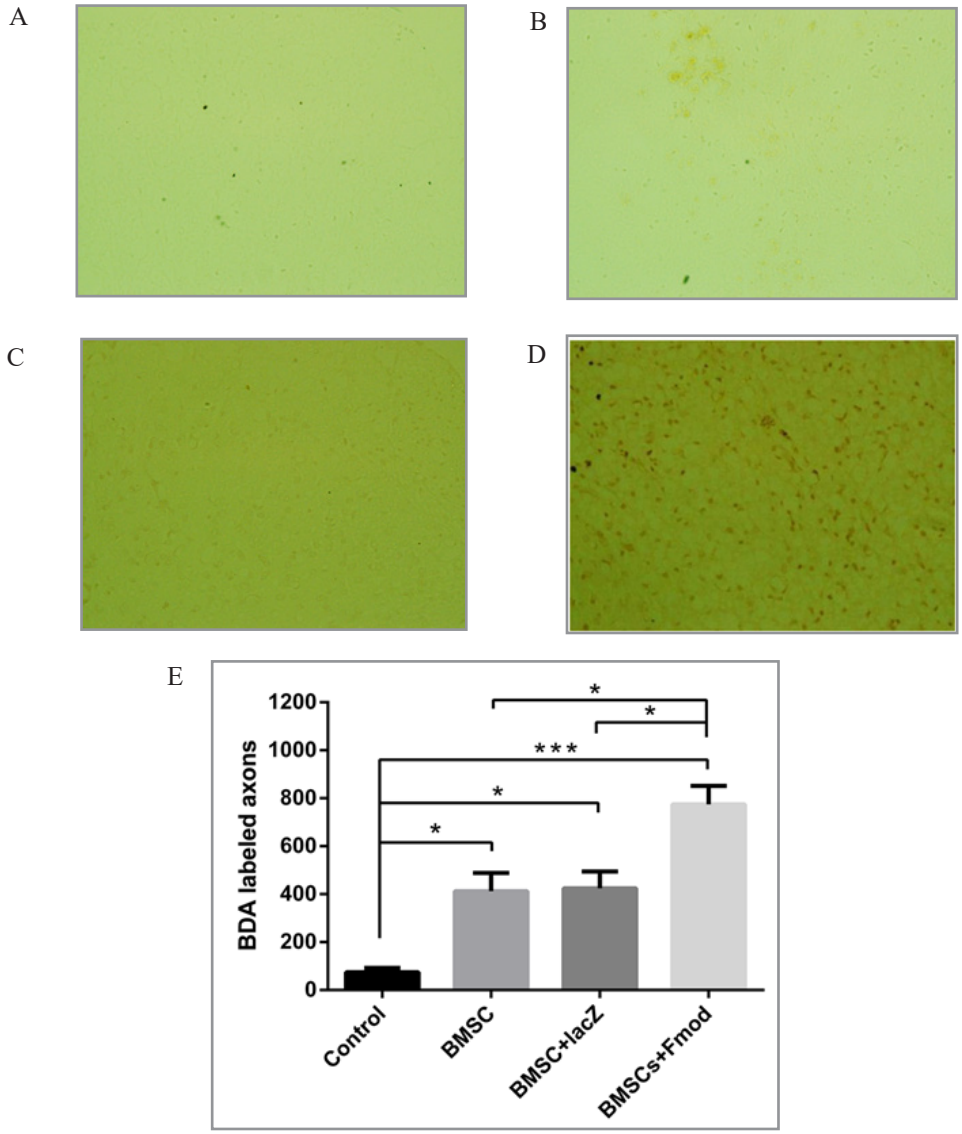

Figure 3: Quantitative Analysis of CST Axons at 6 Weeks After Transplantation:

Effect of BMSCs on axonal regeneration at 6 weeks after spinal cord injury (SCI). (A–D) Measurements of BDA -labeled axons (brown spots) at the lesion site, as were obtained from transversal spinal cord sections from all experimental groups and were presented as numbers of axons (mean ±S.D.). (A) Rats treated with PBS as a control group, (B) rats treated with BMSCs, (C) rats treated with BMSCs infected with adenovirus expressing beta-galactosidase, and (D) rats treated with BMSCs infected with adenovirus expressing Fibromodulin. (E) Quantitative data showing the numbers of BDA-labeled axons per section (n = 12/group). Data were shown as mean ± S.E.M. ***P < 0.001, as compared with the control group. Significant differences were detected in the BMSCs-fibromodulin implanted group compared with the control group (***P<0.001), BMSCs-LacZ implanted group and BMSCs implanted group (*P<0.05). Moreover, there are significant differences between BMSCs-fibromodulin group and BMSCs-LacZ and BMSCs groups (*P<0.05). However, there is no statistical difference between BMSCs-LacZ and BMSCs groups (P value 0.8925).

Discussion

A spinal cord injury (SCI) is a lesion on any part of the spinal cord that leads to short-term or steady-state changes in its normal motor, sensory, or autonomic function. Unfortunately, damaged axons do not generally regenerate and so far, there is no efficient clinically approved strategy to cure SCI [46]. FMOD, a member of the SLRP family, is recognized for its interaction with collagen fibrils and the configuration of the extracellular matrix. Previous studies have reported that FMOD plays a significant role in cell fate determination, fetal-type scarless wound healing stimulates adult wound closure, and decreases scar formation [47-49].

Recent years have seen stem cell transplantation, such as bone marrow mesenchymal cells (BMSCs), embryonic stem cells (ESCs), and umbilical cord blood stem cells, being used to treat

spinal cord injury (SCI). This new strategy has shown promise in activating neuroregeneration and restoring spinal cord functions [50, 51]. BMSCs, in particular, have demonstrated good differentiation potential and neural recovery. They are capable of differentiating into glial cells and neurons, repairing the myelin sheath of injured axons, and regenerating nerve fibers [52]. Additionally, BMSCs produce various trophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), glial cell-derived neurotrophic factor (GDNF), and cytokines such as IL-6 and stem cell factor (SCF), and IGF-1, which are effective in promoting neural protection or regeneration [53, 54]. Transplanted BMSCs produce neurotrophic factors such as BDNF and GDNF that can reduce neuronal cell death protect injured neural tissue and promote axon regrowth, respectively. These factors make BMSC transplantation a promising method for treating SCI. In 2000, Chopp and colleagues demonstrated motor improvement using BMSCs transplantation at the site of the injury in rat models [55]. In 2003, Wu and colleagues investigated bone marrow stromal cell grafting in the lesion site. Transplanted BMSCs stimulated the regeneration of the injured spinal cord by raising tissue repair of the lesion and resulted in smaller cavities than in controls. In another study, Ide and colleagues reduced cavity formation and myelinated injured axons, and increased the BBB score to 9.8 (compared to 5.5-5.7 in the control group) using direct transplantation of BMSCs in the lesion site two weeks post-injury in subacute-spinal-cord injury [56]. To investigate the therapeutic effects of Fibromodulin in combination with BMSCs in a rat spinal cord injury model, the present study transduced BMSCs with adenoviral vectors carrying the Fibromodulin gene. These modified cells were then transplanted into SCI, leading to promoting axonal regeneration and functional recovery. The three BMSCs transplanted groups (BMSCs, BMSCs -LacZ, and BMSCs -Fibromodulin) showed significant axonal regeneration when compared with rats in the control group. This is consistent with previous reports showing that BMSCs injection increases the capability of axon regrowth at the injury site [57-60]. The study found that transplantation of Fibromodulin-expressing BMSCs into the spinal cord four days after injury significantly improved functional outcomes, as evaluated on the BBB test. Significant recovery of functional outcomes extended up to 6 weeks after transplantation. BBB locomotor scaling score results indicated significant scores in weeks 2, 3, 4, 5, and 6 after injection. Behavioral follow-up was performed 6 weeks post-injury. There was no statistical difference until 1 week after injury between groups (p value= 0.325). The average score in the 6th week was 5.60 ±1.140, 8.00, 8.50 ± 0.707, and 9.60 ± 2.675 for groups 1st, 2nd, 3rd, and 4th, respectively. The fourth group had a statistically significant BBB score in comparison with the control group (p value=0.03) in the sixth week, indicating that the Fibromodulin gene had a significant effect on functional recovery. In the third week, the first signs of locomotor function recovery were observed, and they were statistically significant between the fourth and control groups (p value=0.00).

Conclusions

Our study suggests that Fibromodulin may have a significant role in promoting axonal growth after severe injury. As there is no similar study to compare the results with, our focus was to investigate the potential of Fibromodulin for gene therapy of spinal cord injury (SCI) for the first time. Our study also confirmed the positive effect of bone marrow cell therapy combined with gene therapy. Therefore, our findings indicate that the combination of cell therapy (using BMSCs) and gene therapy (using Fibromodulin) can be considered a promising approach for gene therapy of SCI.

Ethics Approval and Consent to Participate

All experimental protocols were undertaken in compliance with the Institutional Animal Care & Use Committee (IACUC) standards and approved by the Zanjan University of Medical Sciences Ethics Review Board (IR.ZUMS.REC.1399. 881).

Consent for Publication

Not applicable. This manuscript does not include any individual person's data in any form (including individual details, images, or videos).

Availability of Data and Material

Data will be made available upon reasonable request.

Competing Interests

The authors declare that they have no conflicts of interest.

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Authors' Contributions

Conception and design: MAK, AB, PR, PAK. Acquisition of data: MAK, MA, IJA. Analysis and interpretation of data: SM, MAK, MA, And AB. Drafting the article: MAK, MA. Critically revising the article: IJA, SM, PR, and PAK. Reviewed submitted version of manuscript: MAK, MA, IJA, SM, PR, PAK, and AB.

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