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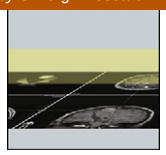

Clinical safety and primary efficacy of bone marrow mesenchymal cell transplantation in subacute spinal cord injured patients

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ABSTRACT

Background: In recent years, some studies were conducted to evaluate the effects of stem cells from different sources on patients with spinal cord injury (SCI). This study was carried out to evaluate the feasibility and therapeutic potential of autologous bone marrow cell (BMC) transplantation in 11 complete spinal cord injured patients at thoracic level.
Methods and materials: This nonrandomized clinical trial compared the results of autologous BMC transplantation into cerebrospinal fluid (CSF) via lumbar puncture (LP) in 11 patients having complete SCI, with 20 patients as control group who received conventional treatment without BMC transplantation. The patients underwent preoperative and follow-up neurological assessments using the American Spinal Injury Association (ASIA) impairment scale. Then, the participants were followed for 12–33 months.
Results: Eleven patients with the mean age of 33.2 ± 8.9 years and 20 patients with the mean age of 33.5 ± 7.2 years were enrolled in the study and in the control group, respectively. None of the patients in the study and control group experienced any adverse reaction and complications, neither after routine treatment nor after cell transplantation. Five patients out of 11 (45.5%) in the study group and three patients in the control group (15%) showed marked recovery, but the result was statistically borderline ($P = 0.095$).
Conclusion: We conclude that transplantation of autologous BMC via LP is a feasible and safe technique, but at the moment, no clear answer can be given regarding the clinical potential, despite a potential tendency to treat SCI patients, observed through statistics.

1. Introduction

Traumatic injury to spinal cord often leads to severe disability in patients and encompasses approximately 150,000–200,000 new spinal cord injury (SCI) cases annually worldwide.

Because of the significant loss of function and financial costs and the fact that previous trials have not demonstrated good results [1], transplantation of a variety of tissues and cells to treat SCI patients has been suggested. Stem cells transplanted into spinal cord lesions may help improve regeneration and spinal cord function. Various types of embryonic stem cells and adult stem cells have been shown to contribute to the axonal regeneration following SCI [2,3].

It has also been suggested that bone marrow cells (BMC) can be used in SCI patients effectively, and recently, it has been used in some clinical studies [4–9].

Although the implantation of stem cells via lumbar puncture (LP) appears to be safe [8–10], fever, headache, and tingling sensation have been reported [8]. Moreover, autologous BMCs would also obviate ethical problems associated with the use of embryonic stem cells [6,7,9,11,12]. The major attraction of BMC transplantation approach is the ease of obtaining patient's autologous tissue at the bedside for transplantation [6]. In addition, BMCs are harvested simply with considerable growth in the culture. These properties make them ideal candidates for cell therapy procedures. Therefore, some investigators suggest that autologous BMC transplantation could be a suitable technique to treat SCI patients. BMCs can be administrated into the injury site [6,13,14] or remote from the injury through either intravenous and intraarterial [15,16] or intrathecal routes [5,8–10,17]. The use of LP for the delivery of stem cells in SCI patients seems to be minimally invasive and allows safe and more efficient delivery of the cells to the injured site of the

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spinal cord [9,10,18]. Only a few clinical trials of cell transplantation in acute and subacute SCI patients have been reported [6,11,19,20]. We performed a clinical trial study for the clinical evaluation of safety and efficacy of the expanded autologous bone marrow transplantation via LP in thoracic and upper lumbar SCI patients within 14–43 days following the onset of injury.

2. Methods and materials

2.1. Patient selection

A nonrandomized phase I/II evaluation of clinical safety and efficacy of autologous BMC in subacute spinal cord injured patients was carried out. The patients in both groups were selected from complete SCI patients (American Spinal Injury Association [ASIA] grade A) who were admitted to Bahonar University Hospital, Kerman, Iran, from December 2006 to August 2009. The stem cell laboratory could service the project only from January 2007 to June 2007 and the patients in the study group were selected consequently in this period of time. The patients in the control group were selected nonconsecutively and randomly before and after this period of time and participants in both groups signed the informed consent form. All the patients in the study and control group received conventional surgery, medical treatment, and rehabilitation.

2.2. Selection criteria

2.2.1. Inclusion criteria

Acute (<2 weeks after SCI) and subacute (2–8 weeks after SCI) patients with complete SCI at thoracic level, ASIA grade A and a contusion confirmed by MR images, age between 10 and 50 years, and injury level from T1–L1 vertebra were the inclusion criteria.

2.2.2. Exclusion criteria

The exclusion criteria included any improvements in the neurological functions before the cell therapy and during the cell preparation period and serious preexisting medical diseases, such as diabetes mellitus, autoimmune diseases, carcinoma, and hypertension.

Standard neurological classification of SCI was performed according to the ASIA protocol [1] to evaluate the neurological deficit. Two neurosurgeons who were unconnected to the study evaluated any neurological improvement in the SCI patients following the intervention. The evaluation was done in both study and control group, and each of the examiners examined the patients in both groups. The follow-up examinations were carried out every three months after the BMC transplantation.

2.3. Preparation of bone marrow stromal cells

All the chemicals were purchased from Sigma Company (Sigma–Aldrich, St Louis, Mo, USA) unless stated otherwise. We used the procedure for the culture of BMCs, which has been described elsewhere, with minor modification [21]. Twenty milliliters of bone marrow were aspirated from the iliac crest and transferred to the cell culture laboratory within 1 h. In the laboratory, the bone marrow was carefully layered on the top of 3 ml of ficoll solution and centrifuged at $350 \times g$ for 10 min. The buffy coat layer at the interface was carefully removed using a fire-polished Pasteur pipette and transferred into a 10-ml sterile tube. The buffy coat was mixed with 5 ml of PBS containing 5.5 mM glucose and centrifuged at $250 \times g$ for 7 min. The cell pellet was removed from the bottom of the tube and transferred into a 75 cm^2 culture flask (Falcon). Subsequently, 15 ml of complete culture medium were added to the flask. The complete culture medium was DMEM–F12 supplemented with 15% FBS (Gibco, UK), 100 IU/ml of penicillin, 60 μg/ml of streptomycin sulfate, and 50 μg/ml of amphotricin B. The culture flasks were incubated at 37 °C humidified atmosphere with 5% CO_2. After 24 h, the floating cells were removed, and the cultures were washed thrice with pre-warmed PBS. Subsequently, 15 ml of DMEM–F12, supplemented with 10% FBS, 1/100 (v/v) insulin–transferrin–selenium solution, and antibiotics, were added to the flask and incubated at the same conditions. The medium was refreshed every four to six days according to the rate of cell propagation. When the attached cells reached >90% confluence, the cells were detached from the substratum with 3 ml of 0.5 g/l trypsin and 0.2 g/l of EDTA. Then, 6 ml of patient's cerebrospinal fluid (CSF) were collected in a sterile tube via LP and transferred to the cell culture laboratory. The BMCs were suspended in 3 ml of the patient's CSF thoroughly and centrifuged at $250 \times g$ for 7 min. The cell pellet was resuspended in 3 ml of the patient's CSF. Subsequently, 20 μl of the suspension were mixed with 20 μl of 0.04% trypan blue, and the number of viable cells was counted with an improved Neubauer chamber. The remaining suspension containing 7×10^5 to 1.2×10^6 viable BMCs was transferred to the operation room for LP administration.

2.4. Adipogenic and osteogenic differentiation of human BMCs

Samples of mononuclear cells were cultured on clean sterile glass slides for 1 week, after which osteogenic and adipogenic differentiations were induced for 14 and 21 days, respectively. The osteogenic medium was DMEM–F12, supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate, 50 μg/ml of ascorbic acid, and 10% FBS. The adipogenic medium was DMEM–F12, supplemented with 100 nM dexamethasone, 50 μg/ml of ascorbic acid, 50 μg/ml of indomethacin, and 10% FBS. The cells cultivated in DMEM–F12 with 10% FBS served as the control. The medium was refreshed every three to four days. The cells were stained with Oil red O and Von-kossa to determine the adipogenic and osteogenic differentiations of the induced cells, respectively.

2.5. Treatment

All the patients were operated to get reduction and fixation and received high dose of methyl prednisolone when applicable. After BMC preparation, 7×10^5 to 1.2×10^6 viable BMCs were injected into CSF via lumbar region at L3–L4 level by LP needle (18G) slowly during about 1 min. Lumbar puncture was performed in the operation room to be sure more about sterility. The BMC-receiving patients and those in the control group underwent neurological examinations every three months.

Both the groups were given supervised physiotherapy (including physical exercise, functional electro-stimulation, ultrasonic diathermy, and infrared) [22] – three hours per day, three days a week – and it continued throughout the study period.

The local ethics committee at Kerman University of Medical Sciences, Kerman, Iran, approved the study protocol (agreement No. K/87/47). The patients were aware of the research project as well as the probable risk factors associated with it. All the procedures were performed after obtaining a written informed consent from the patients. We certify that all the applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during the course of this research.

Fisher's exact test was used to compare the treatment success rate between the study and control groups.

3. Results

3.1. Characterization and differentiation of BMCs

Most of the cells were attached to the substratum shortly after the culture, and the non-adherent cells were discarded during the

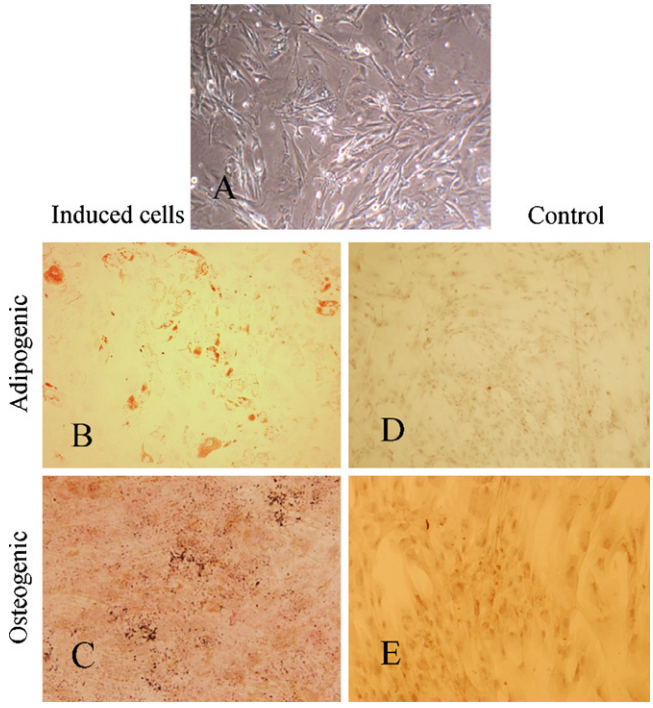

Fig. 1. Human bone marrow cells were cultured to near confluence (A). These cells were induced to differentiate into adipocyte (B) and osteocyte (C) for 14 and 21 days, respectively. Cells in the control group showed few adipocytic (D) and no osteocytic differentiation (E). Original magnification: (A and D) 100× and (B, C and E) 200×.

washing steps. Fibroblast-like cells were observed after 48 h in most of the samples. Other cell types, including fusiform cells – cells with extensive cytoplasmic processes – and roughly round cells were also present in the culture. Nine days after the initiation of adipogenic induction, cells with small lipid vacuoles were detectable when viewed under a phase contrast inverted microscope (Olympus IX70, Japan). The number of adipocytes and lipid vacuoles increased with time. In the control group, very few adipocytes were detected after 14 days. Van-kossa staining revealed calcium phosphate deposits in the extracellular matrix. In addition, osteocyte-like cells with extensive cytoplasmic processes were also observed (Fig. 1). In non-induced cells, no calcium phosphate deposit was detected.

3.2. Patients

Eleven patients, seven males and four females, with mean age of 33.2 ± 8.9 years enrolled in this study. Two patients in the control group refused to participate in the study due to their problem in transportation, therefore, a total of 20 patients, 17 males and three females with mean age of 33.5 ± 7.2 years, were included in the control group. Five cases in the study group and 12 cases in the control group suffered from spinal fracture at T12 and L1 levels, and the others had fracture at other thoracic levels. Both groups were comparable with respect to age ($P = 0.91$), gender ($P = 0.21$) and level ($P = 0.48$). According to the time window between the onset of injury and transplantation (more than two weeks), the patients were considered to be in subacute phase [6]. The majority of the patients in the study and control groups received methylprednisolone, but three cases in the study group and five cases in the control group did not receive methylprednisolone due to a delay in admission to the hospital. Only two cases in the control group underwent decompressive surgery in addition to fixation and fusion. The patients in the study group received autologous BMCs within 27.3 ± 8.4 (range: 14–43) days after SCI. The mean follow-up period in the study and control groups was 20.3 ± 7.2 (range: 12–33) and 23.4 ± 5.6 (range: 12–32) months, respectively. The patients were examined every three months. The major recovery was obtained up to the sixth months, and after that, no major recovery was detected in the patients.

Table 1
Demographic data and the outcome of the case and control groups.

	Study group ($N = 11$)	Control group ($N = 20$)	P value
Mean age	33.18 ± 8.9	33.5 ± 7.16	0.914
Range	23–48	22–48	
Male gender	63.6%	85.0%	0.210
Fracture type			
Fracture dislocation	54.5%	50%	0.571
Burst fracture	45.5%	35%	
Other	0%	15%	
Fracture level			
T1–T11	54.5%	40%	0.477
T12–L1	45.5%	60%	
Prednisolone therapy	72.7%	75%	1.0
Decompressive laminectomy	0%	10%	0.527
Pain	72.7%	65%	1.0
Follow up period	20.3	23.4	0.227
Marked recovery	45.5%	15.0%	0.095

In the cell transplanted group, marked recovery (a two-grade improvement from baseline, i.e. from ASIA A to ASIA C) was reported in five patients (45.5%), when compared with 15% improvement in the control group, reflecting a clinically meaningful improvement, but there was no statistical difference ($P = 0.095$). In these cases, ASIA motor score in lower limb increased from 0 to 19.3 ± 1.5, and the sensory scores increased by 11.5 points, and the patients were able to walk by brace. However, in the control group, three patients improved from grades A to C (15%). None of the patients experienced any detected complications after the routine treatment, such as CSF leakage, infection, or screw malposition. Because of the lack of enough information about the complications of stem cell therapy, we evaluated the reported complication and routine complications of LP after stem cell therapy, so we did not evaluate some unknown complications of stem cell therapy such as urinary tract infection. However, we did not observe any complication in the study and control groups at the operation site. In addition, no detectable complications that could be related to stem cell therapy were found after cell transplantation in two days of hospitalization or long-term follow-up period. Eight patients in the study group and 13 patients in the control group complained of neuropathic pain, yet this difference was not statistically significant. The data from the study and control groups are shown in Table 1.

4. Discussion

The present study demonstrated that 45.5% of the patients at subacute stage showed improved neurological function after autologous BMCs transplantation, which is obviously higher than the rate of improvement in the historical review [23] and our control group. However, despite the differences in the recovery levels between the groups, the small number and heterogeneity of the patients do not allow reliable analysis of the efficacy. We did not find any clinically adverse reaction of cell therapy, such as fever and headache. Pain was seen more frequently in the study group (73%), but it was not significant (Table 1).

Cell transplantation for SCI treatment is a promising therapeutic strategy, and its clinical application would be facilitated by non-invasive delivery procedures. Bakshi reported that BMC delivered by LP reached the contused spinal cord tissues and exerted a significant beneficial effect by reducing cyst and injury size [18]. However,

Table 2
A review of the characteristics and outcome of patients with acute and subacute SCI after autologous BMC transplantation.

	Our study	Park et al. [11]	Yoon et al. [6]	Sykova et al. [16,19]
Mean age	33.18 ± 8.9	36	29.4 ± 13.5	37.5
Range	23–48	17–51	NA	21–41
Injury stage	Sub-acute	Acute	Acute and sub-acute	Acute and sub-acute
Injury level				
Thoracic	11	1	10	1
Cervical	0	5	13	3
Total	11	6	23	4
Time of stem cell therapy after SCI	14–43 days	7 days	2–8 weeks	10–33 days
Cell type	Autologous BMC	Autologous BMC	Autologous BMC	Mononuclear BMC
Cell propagation	Yes	No	No	No
Route of treatment	LP	Injury site	Injury site	IA
Combined therapy	None	G-CSF (subcutaneous)	CM-CSR	G-CSF
Follow up period	12–33 months	6–18 months	10.1–11.3 months	12 months
Improvement				
A to B	0	4 (66%)	NA	
A to C	5 (45.5%)	1 (16.6)	NA	1 (25%)
A to B and C	5 (45.5%)	5 (83.3%)	30.4%	1 (25%)[a]
Complications	None	Fever–myalgia	Fever	–

NA: not available.
[a] Data are obtained from Table 1 (Sykova et al. [16,19]).

we could not find any evidence that shows that LP itself has an effect on the neurological outcome after SCI.

The clinical application of stem cells to treat SCI patients has been reported in the past few years [6,7,9–11,16,24,25]. BMCs have been used in the treatment of hematopoietic diseases for a long time without any immunological problem [26]. Therefore, the use of BMCs would obviate ethical problems concerning the use of embryonic stem cells.

In the present study, the suggestions to conduct clinical trials for SCI patients [1] were fulfilled in a subacute group of thoracic and upper lumbar SCI patients after cell propagation and transplantation via LP. Park et al., in a study group of six SCI patients with lesions at cervical and thoracic levels, reported an improvement from A to B and/or C in five patients after direct BMC transplantation at the injury site combined with subcutaneous injection of G-CSF [11]. Also, in a controlled clinical trial, transplantation of BMCs into the injury site of SCI patients in acute phase resulted in 29.5% neurological improvement from AISA A to B or C, while in the subacute treatment group, 33.3% of the patients improved to AISA B or C [6]. Although we did not administer G-CSF or GM-CSF, the improvement rate in our patients was comparable with that observed by Yoon et al. [6]. However, in the report of Park et al., the improvement rate was much higher (five of six cases) than that of our study as well as the study by Yoon et al. Park et al. limited their study to patients having cervical cord injury in acute phase, which can explain the higher rate of improvement [27]. The results of a multicenter phase 2 study of cell therapy in acute SCI patients have not yet been reported for comparison. In that study, the researchers used invasive method of direct injection of autologous cells. However, they emphasized on less invasive method of intrathechal cell infusion [20]. Data from some reported clinical studies in acute and subacute phases are shown in Table 2 [16].

Due to astrocytic scarring at the injury site, cell transplantation into a chronic SCI is not able to remyelinate the axons [28]. This may stand for the poor results of stem cell transplantation in chronic SCI patients. However, some improvements in chronic SCI patients have been reported after scar removal and transplantation of fetal spinal cord fragment together with olfactory ensheathing cells [12] as well as after transplantation of BMCs, both intravenously and locally into the cyst cavity [14]. Good results have been reported in some studies [4,8,14,15], and the higher rate of improvement in some of these reports [15,24] may be due to the high number of implanted stem cells.

Demyelination of spared axons is a prominent feature of SCI. High number of demyelinating axons has been observed on the first day at the injury epicenter. This demyelination decreases substantially by the seventh day of the injury [28]. In addition to demyelination, SCI also results in a period of secondary degeneration characterized by substantial cellular loss, inflammation, and axonal degeneration. The number of demeylinating axon markedly increases in 120 and 450 days post-injury [28].

Bone marrow stem cells can differentiate into mature neuron or glial cells in vitro as well as in vivo [29,30], may improve the neurological deficits by generating either neural cells or myelin producing cells [31], and can promote axonal regeneration by guiding nerve fibers [32].

The precise mechanism by which transplantation of BMCs promotes functional recovery after SCI is still unclear. One explanation is that factors secreted from the BMCs ameliorate functional deficits [2]. Among these factors are the cytokines that may be neuroprotective and enhance regeneration. Some cytokines, such as Colony Stimulating Factor (CSF), Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), and Vascular Endothelial Growth Factor (VEGF), have been shown to assist nervous tissue regeneration [33]. Remyelination is another possible explanation that has been addressed in some other studies [34].

Sykova et al. recommended "the therapeutic window of 3 to 4 weeks following injury for stem cell treatment [16]. Many other researchers consider 10 to 14 days after SCI as an optimal time period for cell transplantation, but neurologic improvement of a few patients in whom cell transplantation was performed between two and eight weeks after injury has been reported by Yoon et al. [6]. Our results are in agreement with their statement that "the optimal time period of BMC transplantation in SCI patients should not be restricted to patients less than two weeks post-injury."

By the exclusion/inclusion criteria, we tried to decrease the impact of confounding variables, such as age, level of injury, and severity of injury. There was no significant difference between the two groups according to some other confounding factors, such as type of fracture, prednisolone therapy, gender, level, and age (Table 1). The present study was performed on a limited number of SCI patients, which may influence the interpretation of the result, which was statistically borderline. In addition, the strategy requires some time for cell preparation and expansion, which may affect the therapeutic window of the cell transplantation as well as the number of transplanted cells. However, our study had some advantages,

such as patients with complete ASIA A at the thoracic level who are frequently suggested as being the preferred group of SCI patients for the early phase of SCI clinical trials [35]. Our LP route of cell delivery was not time-consuming and invasive, and we did not observe any complications, such as fever and other risks of open surgery.

5. Conclusion

We conclude that BMC grafting via LP is safe; however, at the moment, no clear answer can be given regarding the motor and sensory function of thoracic SCI patients, despite a potential tendency observed through transplantation of BMC. We recommend that the therapeutic value of such treatments should be evaluated in a more comprehensive multicenter study.

Acknowledgments

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