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Effect of Bone Marrow-derived Mesenchymal Stem Cells on Changes of Serum Levels of TNF-α and Locomotor Function after Spinal Cord Injury in Mice

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Spinal Cord Injury (SCI) is still a devastating clinical problem with irreversible consequences leading to permanent functional loss and life time disability. This study was conducted to assess the healing effect of bone marrow-derived mesenchymal stem cells on locomotor function and changes of Tumor Necrosis Factor alpha (TNF-α) after SCI in mice. Forty two BALB/C mice were divided into 3 equal groups of control, SCI and treatment [transplantation of 5×10^4 Bone Marrow Stem Cells (BMSCs)]. The SCI was induced by compression for 2 min at T10 and injury bilaterally. The femoral and tibial bones were used for bone marrow isolation and culture was made using Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum, L-glutamine and penicillin/streptomycin. Cell morphology was evaluated in all passages. Characterization of BMSCs was conducted by reverse transcription polymerase chain reaction and by osteogenic differentiation of BMSCs. The ELISA was undertaken for TNF-α. Open field locomotion was evaluated by Toyama mouse score. The BMSCs were plastic adherent and fibroblastic spindle-shape. MSCs were positive for CD90 and negative for CD34 and CD45. Osteogenic differentiation was noticed when stained with alizarin red. The serum TNF-α level increased after 24 h, 3 and 5 weeks post-SCI and was time dependent. The neurological score significantly improved after 8 weeks after BMSC transplantation. Transplantation of BMSCs was shown to decrease the TNF-α level and inflammation in injured spinal cord and improve the neurological outcome. These findings can be added to the literature for reduction of inflammation in SCI and improvement of neurological outcome after transplantation of BMSCs.

Key words: Bone marrow, mesenchymal stem cells, TNF-a, spinal cord injury, mice

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INTRODUCTION

The relatively permanent nature of Central Nervous System (CNS) injury is an important dilemma, especially if it is related to Spinal Cord Injury (SCI) (Tempel *et al*., 2015). Sports and recreational activities, work-related accidents, motor-vehicle accidents and falls at office or home were reported to be the major causes of trauma to the cord while most of SCI victims are young and otherwise healthy and suffer the burden of a life-long disability (Kwon *et al*., 2011) The annual incidence of SCI was estimated to be about 20-40 persons per million in the world (Cadotte and Fehlings, 2011). The SCI as a serious clinical problem suddenly deprives the patients of neurologic function and decreases their quality of life by developing complications such as chronic pain, spasticity, decubitus ulcers, bowel/bladder dysfunction and compromised sexual function (Scovil *et al*., 2012). The SCI has not only a burden to the concerned patient and their family but also in a great extent to their society by means of socio-economical dropdown of the spinal cord injured patient family, loss of man power to the society and maintenance cost of the patient (Draulans *et al*., 2011).

After SCI, the pathological sequelae are categorized to two broad chronological events of primary and secondary injuries (Oyinbo, 2011). The rst phase is due to a direct mechanical trauma to the spinal cord characterized by axonal, oligodendrocyte and neural cell death (Grossman *et al*., 2001). Accordingly, biologically relevant animal SCI models were focused on development of animal injury models that may reliably mimic human SCI (Chamankhah *et al*., 2013).

Several factors are secreted by astrocytes and microglia leading to an increase in the migration of leukocyte to the site of SCI (Weber *et al*., 2007). Microglias are activated by increasing the level of proin ammatory cytokines, including interleukin-1-α (IL1-α), interleukin-1-β (IL-1β) and Tumor Necrosis Factor-alpha (TNF-α) (Pineau and Lacroix, 2007) TNF-α is a pro-inflammatory, pro-apoptotic cytokine that cause various biological actions, like the induction of apoptosis (Tracey, 2011). The role of TNF-α is extended from the immune system to neuro-inflammatory in the nervous system (Leung and Cahill, 2010). Therefore, there is a need to improve functional recovery of spinal cord after injury including control of inflammation (Dyck *et al*., 2015), rescue of neural tissue (Kwon *et al*., 2013) stimulation of axonal regeneration by modulation of the lesioned environment (Eftekharpour *et al*., 2008) or promotion of remyelination (Gauthier *et al*., 2013).

Currently, the care in the acute phase is often limited to administration of high doses of corticosteroids and surgical stabilization and decompression to possibly attenuate further damages (Fehlings *et al*., 2011). The therapeutic interventions to promote neuronal regeneration may range from genetic modifications and stem cell transplantation to exogenous injection of neuroprotective factors (Tempel *et al*., 2015). A large number of studies were undertaken to determine the effects of transplanting a variety of stem cells or stem cell-derived cells in SCI using different strategies and approaches to address the glial scar and facilitate neuroanatomical plasticity (Eftekharpour *et al*., 2008; Karimi-Abdolrezaee and Eftekharpour, 2012; Alluin *et al*., 2014).

Mesenchymal Stem Cells (MSCs) have been isolated from Bone Marrow (BM) (Aliborzi *et al*., 2015), adipose tissue (Mehrabani *et al*., 2013), umbilical cord blood (Razmkhah *et al*., 2015), endometrial tissue (Ghobadi *et al*., 2015) and menstrual blood (Faramarzi *et al*., 2016). The MSCs have been used for tissue recovery in patients (Mehrabani *et al*., 2016). Multi-lineage properties of MSCs into mesodermal and ectodermal cellular lineages were shown for neuronal-like cells (Jahromi *et al*., 2016), osteoblasts (Aghamir *et al*., 2016) and adipocytes (Mehrabani *et al*., 2013). Bone Marrow Stem Cells (BMSCs) are the most well-known type of mesenchymal stem cells (Mehrabani *et al*., 2016) used based on their anti-in ammatory effects, safety and efficacy in several diseases (Faulkner *et al*,. 2004), such as spinal cord injury (Fehlings *et al*., 2011). This study determined the healing effect of BMSCs on locomotor function and changes of TNF-α after SCI in mice to evaluate the changes in inflammation in SCI and improvement of neurological outcome after cell transplantation.

MATERIALS AND METHODS

Animals: Forty two adult male BALB/C mice (30-35 g) were obtained from the Center of Comparative and Experimental Medicine, Shiraz University of Medical Science, Shiraz, Iran. Animals were randomly divided into 3 equal groups of control (with no intervention), SCI (undergoing SCI) and treatment (transplantation of BMSCs after induction of SCI). They were maintained under standard conditions of temperature of 22±1°C and lighting of 12 h dark/light cycle. They had free access to food and tap water throughout the experiment. This study was approved by the Ethical Committee of Shiraz University.

Spinal cord injury: The SCI was induced as described previously in mice (Faulkner *et al*., 2004) with slight modification. Briefly, mice were anaesthetized with xylazine (Rompun 2%, Bayer 3 mg kg^{-1}) and ketamine (Imalgène 1000, Merial, 30 mg kg^{-1}). Betadine was applied on the skin before a longitudinal incision in the skin was made to expose

the spine between T7-T11 vertebral body levels. Two pairs of forceps attached to a metal frame were used to keep the spine immobilized during the experiments. The spinous process, lamina and pedicles of T10 were removed by a micrometer drill in order to expose the underlying intact dura and spinal cord. The cord was injured at T10 by compression for 2 min bilaterally. Then mice were returned to their cage. After the injury, urinary retention was relieved by twice-daily bladder expressions, as described before (Basso *et al*., 2006).

Isolation and culture of BMSCs: To culture BMSCs, the animals were euthanized and the femoral and tibial bones were removed under sterile condition. After removal of muscular and connective tissues, both ends of the bones were cut and the bone marrow was flushed in a 15 mL falcon tube by a 10 mL syringe full of Dulbecco's Modified Eagle Medium (DMEM; Biovet, Bulgaria) and 1% penicillin streptomycin (Sigma, USA). After isolation of bone marrow; they were kept on ice and under sterile condition and were transferred to stem cell laboratory (Stem Cell and Transgenic Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran).

The bone marrow was diluted with an equal volume of DMEM and centrifuged for 7 min at 1200 rpm. The supernatant was removed and the precipitate was cultured in 25 cm^2 flasks with DMEM supplemented with 10% fetal bovine serum (FBS Biovet, Bulgaria), 1% L-glutamine (Sigma, USA) and 1% penicillin and streptomycin. The culture flasks were transferred into CO_2 incubator with 5% CO_2 at 37°C and saturated humidity. The medium was changed after 24 and then every 3 days until 80% confluency. The adherent cells were then passaged by washing with PBS twice (Gibco, USA) and use of 0.25% trypsin (Gibco, USA) for 3 min. An equal volume of DMEM was used to inactivate the enzyme activity. Cell passaging was continued till passage 3. In cell transplantation group, 5×10^4 BMSCs were administered intravenously via tail 1 day after induction of SCI (5×10^4 cells in a volume of 100 µL of DMEM).

Cell morphology and count: Cell morphology was evaluated using inverted microscope (Olympus, USA).

Characterization of BMSCs by Reverse Transcription Polymerase Chain Reaction (RT-PCR): The RT-PCR conducted to evaluate the expression of markers for MSCs. In summary, after extraction of the total RNA by use of column RNA isolation kit (Denazist-Asia, Iran) based on manufacturer's guideline. It was determined by spectrophotometry. The complementary DNA (cDNA) was purchased from AccuPower Cycle Script RT PreMix Kit (Bioneer, Korea) based on manufacturer's instruction. For each reaction 15 µL of total RNA was used to reach a volume of 20 µL with the DEPC water. Twelve thermal cycles was performed as follows: After 30 sec at 20°C for primer annealing, 4 min at 42°C for cDNA synthesis, 30 sec at 55°C for melting secondary structure and cDNA synthesis and 5 min at 95°C for inactivation.

Then, 1 µL of template (cDNA) and PCR buffer, H_2O, dNTPs, $MgCl_2$, Taq DNA polymerase and forward and reverse primers were mixed. The microtubules containing 20 µL of the mixture were put in thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany) and 30 amplification cycles were done (30 sec denaturation at 95°C, 30 sec annealing at 64, 62 and 61°C and 30 sec extension at 72°C with the 5 min at 95°C for primary denaturation and 5 min at 72°C for final extension). The PCR products were evaluated for defined bands by gel electrophoresis by DNA safe stain in 1.5% agarose gel medium. The bands were visualized by use of UV radiation and a gel documentation system (UVtec, Cambridge, UK) and then were photographed.

Osteogenic differentiation: For osteogenic differentiation, cells from passage 5 were transferred into 6 well plates. At 80% cell confluency, they were plated for 21 days with low glucose DMEM containing 100 nM dexamethasone (Sigma, USA), 0.05l M ascorbate-2-phosphate (Wako Chemicals, USA), 10 mM β-glycerophosphate (Sigma, USA), 1% penicillin/streptomycin and 10% FBS. The medium was replaced every 3 days. After 21 days, osteogenic differentiation was evaluated using alizarin red staining method (Sigma, USA).

ELISA analysis of TNF-α: Six animals in each group were bled after 3 and 5 weeks post-SCI to evaluate the TNF-α serum level. The ELISA tests were performed in a 96-well microtiter plate according to the protocol. The TNF-α mouse *in vitro* ELISA (ab108910) kit was used to assess quantitative measurement of plasma TNF-α level. All materials and reagents were equilibrated to room temperature (18-25°C) prior to use. The plasma was provided using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Then the samples were centrifuged for 10 min at 3000 g. Fifty microliter of TNF-α standard was added to per well and incubated for 2 h. After that the well was washed five times with 200 µL of 1X wash buffer manually.

In next step, each sample was incubated with 50 µL of 1X biotinylated TNF-α antibody for 2 h and washed with wash buffer. The samples were incubated with streptavidin-peroxidase conjugate for 30 min and then unbound conjugates were washed away with wash buffer. To visualize streptavidin-peroxidase enzymatic reaction, chromogen substrate was added per well and incubated till the optimal

blue color product produce. The color was changed into yellow after adding acidic stop solution. The density of yellow coloration was directly proportional to the amount of TNF-α captured in plate.

Locomotor evaluation: For neurological scoring, the mice were separately put in an open-field and observed for 5 min once a week until 8 weeks post-injury. Open field locomotion was evaluated using the 0-8 point Basso Mouse Scale (BMS) score (without tail score), the 0-4 point Body Support Scale (BSS) score and the 0-30 point Toyama Mouse Score (TMS). Animals were allowed to move freely in the plastic box.

Statistical analysis: All quantitative data were evaluated statistically and the significance with one-way ANOVA. The measurement data shall be expressed in (Mean±SD), compared with other groups for significance by one-way analysis of variance (ANOVA) using a statistical software package-Graph Pad Prism and $p<0.05$ indicates that the discrepancy has statistically significance.

RESULTS

Morphology: The BMSCs were plastic adherent and fibroblastic spindle-shape throughout all passages (Fig. 1a-c).

RT-PCR: The BMSCs were positive for CD90 marker of mesenchymal stem cells and negative for CD34 and CD45 markers of hematopoietic stem cells (Fig. 2).

Osteogenic induction: After culture of BMSCs in osteogenic media for 21 days, osteogenic differentiation of the cells were noticed based on presence of calcium deposits when stained with alizarin red (Fig. 3).

ELISA analysis of TNF-α: The effect of BMSCs on proinflammatory cytokine of TNF-α in the injured mouse spinal cord by ELISA demonstrated an increase in the serum TNF-α level after 24 h of post-SCI. The rise in the level of TNF-α was time dependent and more after 35 days in comparison to 21 days post-SCI denoting to a sign of inflammation after SCI. There was a significant decrease in serum level of TNF-α after transplantation of BMSCs in comparison to other groups ($p<0.05$) indicating to the anti-inflammatory and immunomodulatory effects of BMSCs in the injured tissue (Table 1).

Locomotor evaluation: Hindlimb function in mice with SCI mice evaluated by TMS was assessed neurologically every week. The neurological score significantly improved after 8 weeks post-stem cells transplantation ($p<0.05$, Table 2).

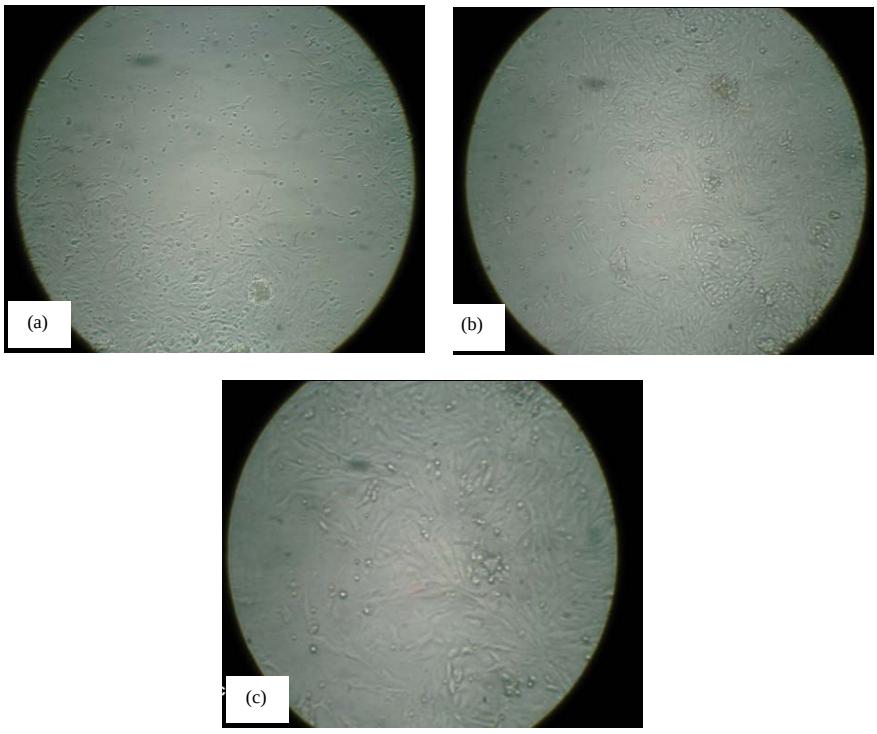

Fig. 1(a-c): BMSCs are plastic adherent and fibroblastic spindle-shape through (a-c) passages

Fig. 2: BMSCs are positive for CD90 marker and negative for CD34 and CD45 markers

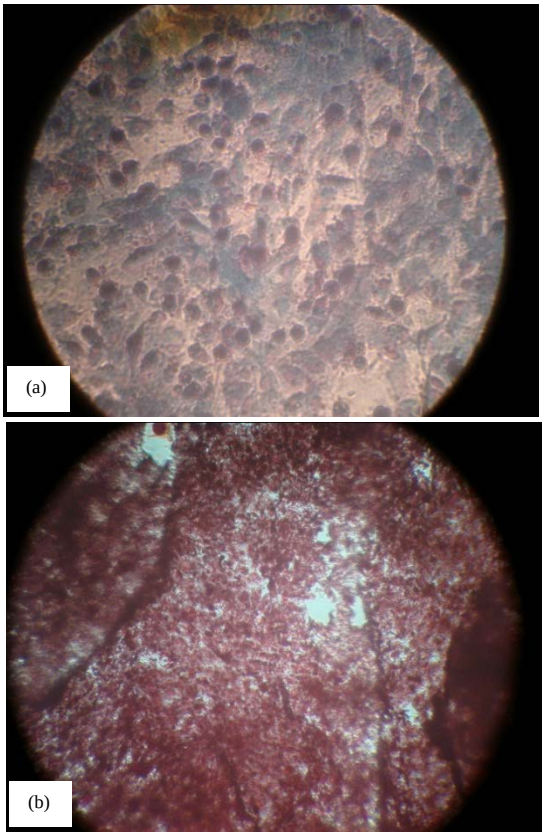

Fig. 3(a-b): Culture of BMSCs in osteogenic media for 21 days, denoting to presence of calcium deposits after staining with alizarin red (a) Control and (b) Osteogenic differentiation

Table 1: Serum TNF–α level in SCI in different time intervals

Groups	Minimum	Maximum	Mean	SD	F	p-value	Sum of squares	df	Mean square
Control (After 3 weeks)	47.90	155.90	97.25	34.49					
Control (After 8 weeks)	67.20	119.70	98.70	14.84					
1 day after lesion	38.50	284.40	164.68	77.61					
3 weeks after lesion	82.90	190.10	139.55	29.48	3.42	0.007	34631.05	6	5771.84
5 weeks after lesion	117.60	147.40	132.50	7.96					
3 weeks after therapy	47.90	197.60	117.72	52.19					
5 weeks after therapy	43.20	136.70	91.40	26.88					

Table 2: Hindlimb function in SCI mice evaluated by transcranial magnetic stimulation (TMS)

Groups	Minimum	Maximum	Mean	SD	F	p-value	Sum of squares	df	Mean square
Control (After 3 weeks)	30.00	30.00	30.00	0.00					
Control (After 8 weeks)	30.00	30.00	30.00	0.00					
3 weeks after lesion	6.00	10.00	7.71	1.60					
5 weeks after lesion	6.00	14.00	9.29	2.98	129.94	0.001	3470.21	5	694.04
3 weeks after therapy	11.00	15.00	13.43	1.72					
5 weeks after therapy	15.00	25.00	20.57	4.19					

DISCUSSION

In this study, administration of BMSCs after SCI could significantly improve the neurological outcomes and diminish the serum level of TNF-α. The serum level of TNF-α increased after SCI due to initiation of inflammation and decreased significantly after 8 weeks with a significant improvement in neurological scores too. The improvement may be due to enhancement in T-cell regulatory activities after transplantation of BMSCs modulating the immune response and reducing the migration of inflammatory cells to lesion area (Lei *et al*., 2015). The MSCs were shown to reduce apoptosis and Caspase 3 activity which could further result in a decrease in neuronal loss after SCI (Hosseini *et al*., 2015).

Also, MSCs can secret GDNF that enhance neuroprotection and decrease inflammation. Transplantation of MSCs can make changes in the secreted cytokines and reduce the secretion of TNF-α, IL-1 and IL-20 (pre-inflammatory cytokines) and increase the TGF-β secretion which regulate inflammatory responses (Piscioneri *et al*., 2015). Another role of MSCs is making the microenvironment enrich for neural regeneration and proliferation of other cells. They can secrete BDNF, bFGF and VEGF in the microenvironment that can promote angiogenesis for survival and proliferation of neural cells. A large population of neurons and glia located in the lesion site undergo death due to the disruption of cell membranes or as a consequence of the ischemia caused by vascular disruption which in turn, causes hemorrhage that extends rostrally and caudally from the lesion site (Yan *et al*., 2001).

The massive cell death extended in the secondary phase occurs by apoptosis and necrosis and affects all functional neurons and glial cell population, including oligodendrocytes (Tator and Koyanagi, 1997). Furthermore, some serum proteins such as thrombin have a neurotoxic effect and can promote additional neural death by themselves or after activating the protease-activated receptor on the microglia (Basso *et al*., 2006; Beattie *et al*., 2002). In the secondary injury process after SCI, apoptosis has been well documented. Oligodendrocytes, microglia and neurons are susceptible to apoptosis. After SCI, some cellular demises were directly related to post-traumatic necrosis, whereas others die due to apoptosis (Sokolova and Reiser, 2008). Spinal cord trauma was shown to activate the upregulation of caspases and calpain and the apoptotic machinery that can lead to an increase in expression of death receptors and their ligands. However, there are conflicting reports as to the role of cell death in SCI- probably a reflection of the known dual capacity of TNF to be both pro and anti-apoptotic (Casha *et al*., 2001).

TNF-α is a proinflammatory and proapoptotic cytokine that can regulate cellular events and contribute to neuronal damage and functional impairment associated with SCI (Harrington *et al*., 2005) TNF-α level is elevated in human spinal cord after SCI, reaching a peak within 1 h after the initial trauma (Dinomais *et al*., 2009) and its expression is upregulated rapidly at the lesion site after SCI (Yan *et al*., 2001) leading to apoptosis of oligodendrocytes and neuronal cell line *in vitro* (Sipe *et al*., 1996). Rapid accumulation of TNF-α may act as an external signal initiating apoptosis after SCI in neurons and glial cells too (Li *et al*., 2000).

The immunomodulatory properties of MSCs have been associated with both molecule secretions and cell-cell contact. It was shown that MSCs were able to suppress T-cell proliferation (Bartholomew *et al*., 2002) and monocyte maturation into dendritic cells (Jiang *et al*., 2005). Moreover, MSCs can impair the functionality of dendritic cells, their antigen-presenting properties and cytokine secretion (Aggarwal and Pittenger, 2005) and also hamper the proper

function of natural killer cells and their interleukin (IL)-2 secretion (Spaggiari *et al*., 2006) that can explain our findings for significant improvements in motor/sensory score.

These improvements were also shown to be correlated with a reduction of in ammatory events of IL-1b and IL-6 expression and an increase in IL-10 expression. Moreover, the number of activated macrophages was demonstrated to be reduced in these conditions (Seo *et al*., 2011). These findings are in consistent with previous observations from Abrams *et al*. (2009) too showing that BMSCs transplantation could decrease the astrocytic reactivity and microglial activation inside the lesioned spinal cord, associated with a reduced injury-induced response to mechanical stimuli.

The MSCs transplantation were shown to be associated with a reduction in IL-6 and TNF-α levels at the lesion site. All these events are correlated with a signi cant recovery of locomotor function in mice SCI (Nakajima *et al*., 2012). Although, PGE2 might be an intriguing candidate factor to be released by MSCs after cell-to-cell contact. Aggarwal and Pittenger (2005) proved that the inhibition of PGE2 synthesis restored the secretion of TNF-α and IFN-γ by dendritic cells cultured in the presence of MSC.

CONCLUSION

Our results denoted to the point that transplantation of BMSCs can decrease the TNF-α level and inflammation in injured spinal cord tissue and further improve the neurological outcome. These findings can add the literature in reduction of inflammation in SCI and improvement of neurological outcome after transplantation of BMSCs.

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