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Brief Report

Impacts of Bone Marrow Stem Cells on Caspase-3 Levels after Spinal Cord Injury in Mice

Noushin Gashmardi[1,2], PhD Candidate;
Seyed Ebrahim Hosseini[2], PhD;
Davood Mehrabani[3], PhD;
Mohammad Amin Edalatmanesh[2], PhD;
Zahra Khodabandeh[3], PhD

[1]Department of Physiology, College of Sciences, Fars Science and Research Branch, Islamic Azad University, Fars, Iran;
[2]Department of Physiology, College of Sciences, Shiraz Branch, Islamic Azad University, Shiraz, Iran;
[3]Stem Cell Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Correspondence:
Seyed Ebrahim Hosseini, PhD;
Department of Physiology, College of Sciences, Shiraz Branch, Islamic Azad University, Shiraz, Iran
Tel: +98 71 3112201
Email: ebrahim.hossini@yahoo.com
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Abstract

Spinal cord injury (SCI) is a drastic disability that leads to spinal cord impairment. This study sought to determine the effects of bone marrow stem cells (BMSCs) on caspase-3 levels after acute SCI in mice. Forty-two mice were randomly divided into 3 groups: control (2 subcategories), subjected to no intervention; sham (3 subcategories), subjected to acute SCI; and experimental (2 subcategories), subjected to SCI and cell transplantation. In the experimental group, 2×10^5 BMSCs were injected intravenously 1 day after SCI. The mesenchymal property of the cells was assessed. The animals in the 3 groups were sacrificed 1, 21, and 35 days after the induction of injury and caspase-3 levels were evaluated using a caspase-3 assay kit. The obtained values were analyzed with ANOVA and Tukey tests using GraphPad and SPSS. Based on the assessments, the transplanted cells were spindle-shaped and were negative for the hematopoietic markers of CD34 and CD45 and positive for the expression of the mesenchymal marker of CD90 and osteogenic induction. The caspase-3 levels showed a significant increase in the sham and experimental groups in comparison to the control group. One day after SCI, the caspase-3 level was significantly higher in the sham group (1.157±0.117) than in the other groups (P<0.000). Twenty-one days after SCI, the caspase-3 level was significantly lower in the experimental group than in the sham group (0.4±0.095 vs. 0.793±0.076; P<0.000). Thirty-five days following SCI, the caspase-3 level was lower in the experimental group than in the sham group (0.223±0.027 vs. 0.643±0.058; P<0.000). We conclude that BMSC transplantation was able to downregulate the caspase-3 level after acute SCI, underscoring the role of caspase-3 as a marker for the assessment of treatment efficacy in acute SCI.

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Keywords ● Bone marrow transplantation ● Mesenchymal stromal cells ● Caspase-3 ● Spinal cord injuries ● Mice

What's Known

- Cell transplantation in the healing of acute spinal cord injury (SCI) has always been considered an important and challenging issue.
- Changes in gene expression for caspase-3 after cell transplantation have been studied; however, there is always a need for a simple and inexpensive method to determine caspase-3 levels.

What's New

- Effects of cell transplantation on caspase-3 levels in acute SCI as a marker for the efficacy of treatment were determined using available ELISA kits and the results showed that mesenchymal stem cells reduced apoptosis and prevented its continuity.
- Caspase-3 can be used quantitatively for the assessment of apoptosis in acute SCI.

Introduction

Spinal cord injury (SCI) is a drastic disability which might lead to complete or incomplete spinal cord impairment. Road accidents, falls, and sports have been shown as the prevalent reasons for SCI. In the wake of SCI, a cascade of neurotoxins triggers local inflammatory responses and apoptosis.[1] Apoptotic pathways can be either intrinsic or extrinsic. The intrinsic pathway is begun by changes in mitochondrial permeability and the activation of caspase-9 and caspase-3.[2] Caspase-3 is an apoptotic marker

that can be activated after traumatic SCI and is first detected in neurons and afterward in oligodendroglial cells.[3] Apoptosis may strongly cause the paralysis of patients with SCI. Therefore, reducing apoptosis may decrease secondary degeneration and functional imperfection after SCI. The treatment of SCI requires that the damaged cells be repaired and regenerated.[4] Mesenchymal stem cells (MSCs) have been successfully used in the treatment of SCI.[5] Several studies have shown that bone marrow stem cells (BMSCs) may have a protective role in central nervous system (CNS) damage by secreting trophic and soluble factors in neurons and oligodendrocytes, thus activating the endogenous survival of signaling pathways.[6] Steward et al.[7] (1999) demonstrated a large difference between mice and rats in their responses to CNS injury for the fabrication of neurotrauma research over time including animal size, bone structure, and cellular responses (inflammation, apoptosis, and necrosis). So far, there have been relatively few studies on SCI in mice. Accordingly, the current study was performed to determine the effects of BMSCs on caspase-3 levels after SCI in an experimental mouse animal model.

Materials and Methods

Experimental Animals

Forty-two male BALB/C mice, weighing between 30 and 35 g, were purchased from the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. The mice were kept under standard conditions (temperature: 22±1 °C, time light and dark: 12 h: 12 h). Sufficient amounts of food and water were freely available. All the trials on the animals were approved by the Ethics Committee of Islamic Azad University based on the rules for working with laboratory animals of Iran Veterinary Organization (ethical code: miau.ir.l396.2400).

The animals were randomly divided into 3 groups: 1) control (n=12), subjected to no intervention; 2) sham (n=18), subjected to SCI; and 3) experimental group (n=12), subjected to SCI and then cell transplantation. The animals in the control and experimental groups were divided into 2 subgroups, followed up after 21 and 35 days. The 18 animals in the sham group were divided into 2 subgroups and were evaluated 1, 21, and 35 days post-SCI. In the experimental group, 2×10^5 BMSCs were injected intravenously, 1 day after the injury. The animals in each group were sacrificed on different days to evaluate the level of caspase-3 using an ELISA kit. The time points of day 1 (n=6), day 21 (n=18), and day 35 (n=18) were selected to approximately match the peaks of caspase-3.

SCI Model

The mice were anesthetized with xylazine (2% Rompun, Bayer Co., Germany, 3 mg/kg) and ketamine (Imalgène 1000, Merial, Germany, 30 mg/kg) intraperitoneally. After the prepping of the back region with betadine, a longitudinal incision was made over the T7–T11 region. The vertebral column was exposed, and a micrometer drill was used until the intact dura was observed. SCI was induced at T10 by compression for 2 minutes bilaterally.[8] Afterward, the incision area was sutured and the mice were returned to their cages. Twice per day, the bladder was emptied manually.

Isolation and Culture of the BMSCs

MSCs were harvested from the femoral and tibial bones of the adult mice. The bone marrow was aspirated and was then diluted with an equal volume of DMEM. Centrifugation was undertaken for 7 minutes at 1200 rpm, while the supernatant was removed. The precipitate was seeded into plastic culture flasks containing DMEM supplemented with 10% fetal bovine serum (FBS; Biovet, Bulgaria), 1% L-glutamine (Sigma, USA), and 1% penicillin and streptomycin. The culture flasks were left in a CO_2 incubator with 5% CO_2 at 37 °C and saturated humidity.

Characterization of the BMSCs by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The expressions of CD90 for mesenchymal and CD34 and CD45 for hematopoietic markers were evaluated via RT-PCR. In brief, a column RNA isolation kit (Denazist-Asia, Iran) based on the manufacturer's guideline was used for the extraction of total RNA, which was then assessed by spectrophotometry. The complementary DNA (cDNA) was prepared based on the manufacturer's instruction from AccuPower Cycle Script RT PreMix Kit (Bioneer, Korea). Thereafter, 1 µL of template (cDNA) and PCR buffer, dNTPs, MgCl2, H_2O, Taq DNA polymerase, and forward and reverse primers were mixed. The microtubules contained 20 µL of the mixture and were transferred in a thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Germany). The PCR products were assessed for defined bands by gel electrophoresis and DNA safe stain in a 1.5% agarose gel medium. The bands were visualized using UV radiation and a gel documentation system (UVItec, Cambridge, UK) before photography was undertaken.

First primers were designed as follows: for CD90, the forward primer sequences were GAAGACAAGGAGCCAGAAC and the reverse was GCAAGGGAAAGAAGAATAAAGG (118 bp). The data for CD34 were AATGAGTCTGTTGAGGAA and CTGTCTGAAGTAGTAGGC (215 bp) and for CD45 were AAGTGGATGTCTATGGTTA and GAAGGAAGTCTCTGGTAT (226 bp), respectively.

Osteogenic Differentiation

The osteogenic differentiation property of the BMSCs was determined by seeding the cells at passage 3 and 80% confluency into 6-well plates for 21 days adding glucose DMEM supplemented with 100 nM of dexamethasone (Sigma, USA), 0.05l M of ascorbate-2-phosphate (Wako Chemicals, USA), 10 mM of b-glycerophosphate (Sigma, USA), 1% penicillin/streptomycin, and 10% FBS. After 21 days, osteogenic differentiation was assessed using the Alizarin Red staining method (Sigma, USA).

Caspase-3 Activity Assay

Six mice from each group were sacrificed 1 day post-SCI and also after 3 and 5 weeks post-SCI to evaluate the activity of caspase-3 using an assay kit (ab39401, Colorimetric) according to the company's instructions.

Statistical Analysis

GraphPad (version 6.03) and SPSS (version 23.0, Chicago, IL, USA) were employed for the statistical analyses. The data on the changes in the caspase-3 level are presented as mean±SD. The comparisons were done using one-way analysis of variance (ANOVA) and the Tukey test. A p value less than 0.05 was considered statistically significant.

Results

Morphology of the BMSCs

The BMSCs were fibroblastic and spindle-shaped throughout all the passages (figure 1).

Characterization of the BMSCs by RT-PCR

The BMSCs were positive for CD90 (MSC marker) and negative for CD34 and CD45 (hematopoietic stem cell markers) (figure 2).

Osteogenic Induction

The osteogenic differentiation of the BMSCs in the osteogenic media after 21 days was positive and was confirmed by the presence of calcium deposits stained with Alizarin Red (figure 3).

Caspase-3 Level Assay

The descriptive statistics of the caspase-3 levels in all the groups are presented in table 1. Caspase-3 significantly increased in the sham and experimental groups when compared to the control group and was significantly higher in the sham group than in the other 2 groups after 1, 21, and 35 days (table 1 and figure 4).

A decrease in the caspase-3 level was noticed following BMC transplantation in the animals with SCI. Twenty-one days post SCI, the caspase-3 level was significantly lower in the experimental group than in the sham group (0.4±0.095 vs. 0.793±0.076; P<0.000). Thirty-five days after SCI, the caspase-3 level was significantly lower in the experimental group than in the sham group (0.223±0.027 vs. 0.643±0.058; P<0.000). One day following SCI, the caspase-3 level was significantly higher in the sham group (1.157±0.117) than in the

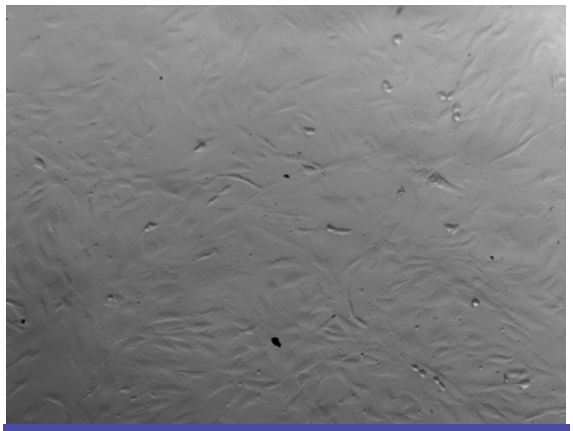

Figure 1: Morphology of the bone marrow stem cells shows spindle-like morphology at passage 3.

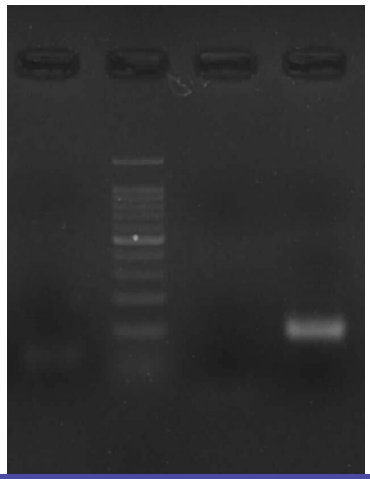

Figure 2: Expression of the markers for the mesenchymal stem cells (CD90+: 118 bp) and the absence of expression for the hematopoietic stem cells (CD34-, CD45-) in passage 3 (P3) of the bone marrow stem cells using reverse transcription polymerase chain reaction (markers: 215 and 226 bp, respectively).

Table 1: Caspase-3 level in the different groups at various time intervals

Groups			Mean difference & P value						
			1	2	3	4	5	6	7
1	Control (after 3 wk)	(0.053±0.008)		0.004 (1.000)	–1.104 (0.001)	-0.741 (0.001)	–0.591 (0.001)	–0.347 (0.001)	–0.171 (0.002)
2	Control (after 5 wk)	(0.049±0.005)	–0.004 (1.000)		–1.108 (0.001)	–0.745 (0.001)	–0.595 (0.001)	–0.351 (0.001)	–0.175 (0.002)
3	Sham (1 d after SCI)	(1.157±0.117)	1.104 (0.000)	1.108 (0.000)		0.363 (0.000)	0.513 (0.000)	0.757 (0.000)	0.933 (0.000)
4	Sham (3 wk after SCI)	(0.793±0.076)	0.741 (0.000)	0.745 (0.000)	–0.363 (0.000)		0.150 (0.009)	0.393 (0.000)	0.570 (0.000)
5	Sham (5 wk after SCI)	(0.643±0.058)	0.591 (0.000)	0.595 (0.000)	–0.513 (0.000)	–0.150 (0.009)		0.243 (0.000)	0.420 (0.000)
6	Experimental (3 wk after SCI)	(0.4±0.095)	0.347 (0.000)	0.351 (0.000)	–0.757 (0.000)	–0.393 (0.000)	–0.243 (0.000)		0.177 (0.001)
7	Experimental (5 wk after SCI)	(0.223±0.027)	0.171 (0.002)	0.175 (0.002)	–0.933 (0.000)	–0.570 (0.000)	–0.420 (0.000)	–0.177 (0.001)	

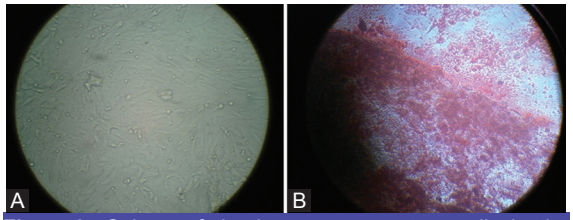

Figure 3: Culture of the bone marrow stem cells in the osteogenic media after 3 weeks leads to the osteogenic differentiation of the cells using Alizarin Red staining based on the presence of calcium deposits in the differentiated cells (A: Control, B: Osteogenic induction).

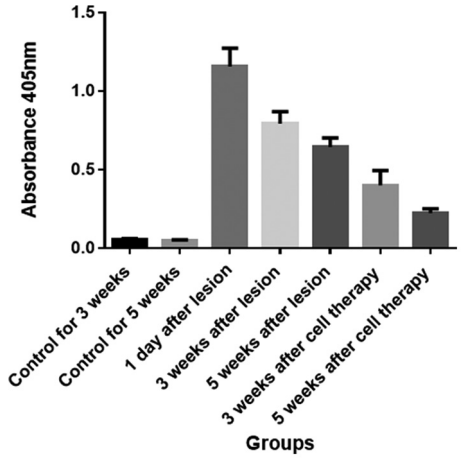

Figure 4: Decrease in the caspase-3 level after cell transplantation in the experimental group in comparison to the sham group.

other groups (P<0.000). The results of ANOVA revealed significant differences between the groups regarding the caspase-3 level ($F_{6,35}$=219.807; P<0.001).

Discussion

The results of the present study showed that the BMSCs were spindle-shaped, suggesting that they were mesenchymal, which is similar to the results of previous investigations.[9] The ability of the BMSCs to differentiate into osteoblasts was confirmed based on the presence of calcium phosphate and Alizarin Red staining.[10] What also exhibited the mesenchymal feature of the isolated cells was that the markers were positive for CD90 and negative for CD34 and CD45.[9] In the present study, the caspase-3 level increased significantly in the sham and experimental groups receiving BMSCs when compared to the control group. A decreasing trend was seen after cell transplantation when compared to the sham group, which may have been due to the effects of BMSCs on apoptosis in SCI.

Caspases are proteolytic enzymes that can cause cell death by apoptosis, necrosis, and inflammation. However, they have non-apoptotic roles including proliferation, tumor suppression, and aging too. Following SCI, neuronal and oligodendroglial apoptosis contributes to demyelination and axonal degeneration and the level of caspase-3 increases in both neurons and glial cells, and thus death receptors and their ligands expresse.[11]As caspase-3 activity is an indicator of apoptosis,[12] the activity of caspase-3 after SCI may lead to cell dysfunction or death. The activation of caspase-3 happens shortly after injury. The increase in the caspase-3 level subsequent to SCI denotes its vital role in cell death as a result of damage to the spinal cord. Neurological failure in the axonal injury of SCI happens due to lack of conduction in the nerves.

CNS injury leads to damage to the blood–brain barrier and infiltration of peripheral immune cells, which might increase both apoptosis and necrosis. Sun et al.[13] (2017) demonstrated that in spinal cord ischemia reperfusion, the calcium-sensing receptor is activated, stimulating apoptosis. Calpain, also known as calcium-dependent protease system, is an effective

molecule reacting to an intracellular calcium signal, calpain-1, which has been shown to selectively cleave some apoptotic proteins (e.g. caspase-3) and thus regulate apoptotic cell death (Ca directly or by activation of calpain-1, leading to apoptosis).[14]

Cell transplantation in SCI has always been attractive for researchers. The results of a study showed that BMSCs have the ability to repair oligodendrocytes, to prevent progressive myelin loss, and to induce remyelination and axonal regeneration.[15] BMSCs can inhibit apoptosis by replacing lost cells, especially oligodendrocytes, and as such facilitate the remyelination of spared axons. In the treatment of SCI, the protection of cells by a decrease in the secondary injury process has been demonstrated. MSCs have been shown to reduce apoptosis and caspase-3 activity, which could decrease neuronal loss in the wake of SCI. Also, MSCs contribute to the microenvironment enriched neural regeneration and proliferation. They can secrete BDNF, bFGF, and VEGF in the microenvironment, which can promote angiogenesis for the survival and proliferation of neural cells.[16]

In the present study, the BMSCs appeared to have neuroprotective effects and to decrease the apoptotic processes by downregulating caspase-3 and other extrinsic pathway proteins during secondary damage to SCI. The downregulation of the Fas receptor protein and caspase-3 can mediate apoptotic proteins after SCI and promote the survival of neurons and oligodendrocytes.[17] In previous studies, we showed that transplanted stem cells promoted significant functional recovery after SCI.[5] BMSCs are suggested to have the capacity to differentiate into neural lineages. In brain ischemia, BMSCs have been shown to secrete supportive substances such as neurotrophins and growth factors, which may have permanent beneficial effects on the tissue. On the other hand, stem cells can provide new cells to heal the injured tissue following SCI.

Hu et al.[17] (2010) reported that MSC transplantation could cause an increase in hematopoiesis and a decrease in apoptosis. Changes in caspase-3 levels in the nervous system have previously been investigated in SCI due to ischemia/reperfusion injury, neuropathic pain due to chronic constriction injury, and Parkinson's and Alzheimer's diseases. Hyperbaric oxygen has been demonstrated to decrease caspase-3 and inhibit inflammation and apoptosis after SCI. Following SCI, the caspase-3 pathway undergoes changes, indicative of its importance in the treatment of the disease.[18] The results of our study revealed that the MSCs reduced apoptosis and caspase-3 activity and they could further result in a decrease in neuronal loss after SCI.

First and foremost among the limitations of the present study is that we did not differentiate apoptosis separately for oligodendrocytes and neurons. Another limitation of note is the lack of information on anti-apoptosis in the studied cells. The fact that we did not use real-time PCR to assess gene expressions for bax and bcl2 and their ratio is another limitation. Nonetheless, the strength of our study is that we defined the effects of changes in the caspase-3 level as a marker for the efficacy of cell transplantation in SCI in a mouse model by using available ELISA kits.

Conclusion

We showed that BMSC transplantation decreased caspase-3 and apoptosis after acute SCI, denoting the role of caspase-3 as a marker for the assessment of treatment efficacy in acute SCI.

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Conflict of Interest: None declared.

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