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RESEARCH ARTICLE

12 hours after cerebral ischemia is the optimal time for bone marrow mesenchymal stem cell transplantation

Seyed Mojtaba Hosseini[1, 2, 3, *], Mohammad Farahmandnia[1, 2], Zahra Razi[1, 2], Somayeh Delavarifar[1, 2], Benafsheh Shakibajahromi[1, 2]

1 Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran
2 Cell and Molecular Medicine Student Research Group, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran
3 Stem Cell Laboratory, Department of Anatomy, Shiraz University of Medical Sciences, Shiraz, Iran

***Correspondence to:**
Seyed Mojtaba Hosseini, M.D., hoseini2010m@gmail.com.

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Abstract

Cell therapy using stem cell transplantation against cerebral ischemia has been reported. However, it remains controversial regarding the optimal time for cell transplantation and the transplantation route. Rat models of cerebral ischemia were established by occlusion of the middle cerebral artery. At 1, 12 hours, 1, 3, 5 and 7 days after cerebral ischemia, bone marrow mesenchymal stem cells were injected *via* the tail vein. At 28 days after cerebral ischemia, rat neurological function was evaluated using a 6-point grading scale and the pathological change of ischemic cerebral tissue was observed by hematoxylin-eosin staining. Under the fluorescence microscope, the migration of bone marrow mesenchymal stem cells was examined by PKH labeling. Caspase-3 activity was measured using spectrophotometry. The optimal neurological function recovery, lowest degree of ischemic cerebral damage, greatest number of bone marrow mesenchymal stem cells migrating to peri-ischemic area, and lowest caspase-3 activity in the ischemic cerebral tissue were observed in rats that underwent bone marrow mesenchymal stem cell transplantation at 12 hours after cerebral ischemia. These findings suggest that 12 hours after cerebral ischemia is the optimal time for tail vein injection of bone marrow mesenchymal stem cell transplantation against cerebral ischemia, and the strongest neuroprotective effect of this cell therapy appears at this time.

***Key Words:** nerve regeneration; bone marrow mesenchymal stem cells; cerebral ischemia; tail vein injection; middle cerebral artery occlusion; cell therapy; neuroprotection*

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Introduction

Stroke is one of the most important central nervous system disorders, which is caused by hypoperfusion and has serious consequences such as various disabilities in the patient's whole life without any curative treatments (Lindvall and Kokaia, 2006). Thus, the reduction of pro-inflammatory cytokines could diminish the secondary damage and apoptosis. An excessive amount of apoptosis is seen in neurodegenerative diseases including brain stroke (Ko et al., 2009; Sung et al., 2012; Kim et al., 2014). Post-cerebral ischemia apoptosis plays an important role in neural death and secondary damage that is not a direct result of the hypoperfusion (Broughton et al., 2009). A major factor of initiating apoptosis is to activate caspase-3, which can trigger the apoptosis cascade (Fan et al., 2005).

Nowadays, stem cell therapy has opened a new window for central nervous system diseases due to the regenerative capacity and anti-inflammatory effects of stem cells. Mesenchymal stem cells (MSCs) are a group of available stem cells isolated from different sources such as bone marrow and adipose tissue (Bang et al., 2005). Immunomodulatory and poorly immunogenic properties of MSCs make them a good source for cell therapy (Sung et al., 2012). MSCs exert immunomodulatory effects by diminishing the production of adhesion molecules and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), and activating T helper cells (Bartholomew et al., 2002; Le Blanc et al., 2003).

The anti-inflammatory, immunomodulatory and anti-apoptotic properties of MSCs make them a good candidate for treating central nervous system diseases such as brain stroke. The aim of this study was to investigate the optimal time for MSCs injection against cerebral ischemia and reveal the neuroprotective effects of MSCs in rat modes of cerebral ischemia by assessing neurological function recovery and determining infarct volume and caspase-3 activity (apoptosis) in ischemic cerebral tissue at 1, 12 hours, 1, 3, 5 and 7 days after cerebral ischemia induction.

Materials and Methods

The rats used in this study were supplied from Animal Laboratory of Shiraz University of Medical Sciences, Iran. The rats had free access to food and water. All procedures were done according to the guidelines of Shiraz University of

Medical Sciences Ethical Committee, Iran.

Experimental design

120 adult male rats (250–300 g) were randomly selected and divided into eight groups (n = 15): ischemia group (only MCAO), PBS group (MCAO + 200 μL PBS), and cell transplantation groups (receiving MSCs *via* the tail vein at 1, 12 hours, 1, 3, 5 and 7 days after MCAO).

Establishment of middle cerebral artery occlusion (MCAO) models in rats

Occlusion of the middle cerebral artery was performed according to the method as previously described (Jin et al., 2010). Briefly, the rats were anesthetized by halothane inhalation (5% for induction and 2% for maintenance) and then, a vertical incision was made in the midline of the neck. The right common carotid, internal carotid and external carotid arteries were exposed and separated from the vagus nerve. Two loose sutures were prepared below carotid bifurcation and the external carotid artery was clamped. A silicone-coated nylon suture 4.0 was inserted into the common carotid artery through a little incision made at the external carotid artery. Thirty minutes later, the nylon suture was removed and the sutures were tightened up so that the blood could flow *via* the external carotid artery by removing the clamp (Jin et al., 2010).

MSCs injection

The MSCs used were isolated from bone marrow of male Sprague-Dawley rats (8 weeks old, 250–300 g) according to a previously described method (Lindvall O and Kokaia Z, 2006). Rat femoral and tibial marrow was harvested by flashing the femur and tibia using complete media (Dulbecco's modified Eagle's medium/F12, 10% fetal bovine serum and 1% penicillin-streptomycin), and then MSCs isolated from femoral and tibial marrow were transferred to a T-25 cm^2 cell culture flask. Subsequently, MSCs were washed with PBS and the culture medium was refreshed once every 2 days. When cells reached 80% confluence at 1 week after seeding, they were passaged and then transferred to two new flasks. Under the inverted microscope (Olympus, Tokyo, Japan), MSCs were processed immunocytochemically for CD44 (ab157107, 1:200; Abcam, Cambridge, MA, USA), CD90 (ab225, 1:1,000; Abcam), CD29 (04-1109, 1:250; Millipore, Billerica, MA, USA) and CD34 (ab185732, 1:200; Abcam) (Lindvall and Kokaia, 2006). Passage 4 MSCs (2.0×10^5) were diluted in 200 μL PBS and then injected into the rats *via* the tail vein. 4 μL of PKH-26 (a lipophilic marker) solution (Sigma, St. Louis, MO, USA) diluted with diluents C, which was supplied by the PKH kit (Sigma), was used to trace the MSCs injected *via* the tail vein (Horan and Slazak, 1989).

Assessment of neurological function

At 28 days after MCAO, rat neurological function was scored using a 6-point grading scale (Del Zoppo et al., 2000). 0, No neurological deficits; 1, failure to extend the left forepaw completely (mild focal neurological deficit); 2, circling to the left (moderate focal neurological deficit); 3, falling to the left (severe focal neurological deficit); 4, no spontaneous walking and decreased level of consciousness; 5, death due to cerebral ischemia.

Hematoxylin-eosin staining

At 28 days after MCAO, rats were sacrificed with deep anesthesia and perfused with normal saline followed by 4% paraformaldehyde. The brain specimens were prepared, cut into 10 μm thick coronal sections, and then mounted on silicon pre-coated slides. The sections were selected with 1 mm intervals and the first one being 2 mm posterior to the frontal pole. Then these sections were stained with hematoxylin-eosin and observed under the inverted microscope (Olympus, Japan) (Del Zoppo et al., 2000).

Cell distribution

Distribution of transplanted cells, *i.e.*, PKH-labeled cells, in peri-infarct area was observed by fluorescent microscopy (Olympus, Germany).

Evaluation of apoptosis by measurement of caspase-3 activity

Activation of interleukin-1β-converting enzyme (ICE) family proteases/caspases initiates apoptosis in mammalian cells (Brentnall et al., 2013). The assay that measures caspase-3 activity is based on spectrophotometric detection of chromophore p-nitroaniline (*p*-NA) after cleavage from labeled substrate DEVD-*p*-NA. The *p*-NA light emission could be measured using a spectrophotometer (Eppendorf; Germany) at 400–405 nm. For this assay, caspase-3 assay (Abcam, UK, cat. No ab39401) kit was used (Yilmaz G and Granger DN, 2010). The activity of caspase-3 in the ischemic brain tissue of the right hemisphere of rats in each group was measured at 1, 7, 14, 21 and 28 days after MCAO.

Statistical analysis

SPSS 18.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Intergroup differences in neurological scores, distribution of transplanted MSCs, and caspase-3 activity were analyzed using one-way analysis of variance. Intergroup difference in brain infarct volume was analyzed using the Kruskal-Wallis test, a nonparametric test.

Results

Characterization of bone marrow MSCs (BMSCs)

After 1 week after injection, BMSCs reached 80% confluence and exhibited a fibroblast-like phenotype. They presented with a small cell body with a few thin and long cell processes as shown under the inverted microscope (**Figure 1**). BMSCs were positive for CD44 (97.5 ± 1.32%), CD90 (98.0 ± 1.73%), and CD29 (97.5 ± 0.70%) and they hardly expressed hematopoietic or endothelial marker CD34 (2.2 ± 0.87%) (**Figure 2**).

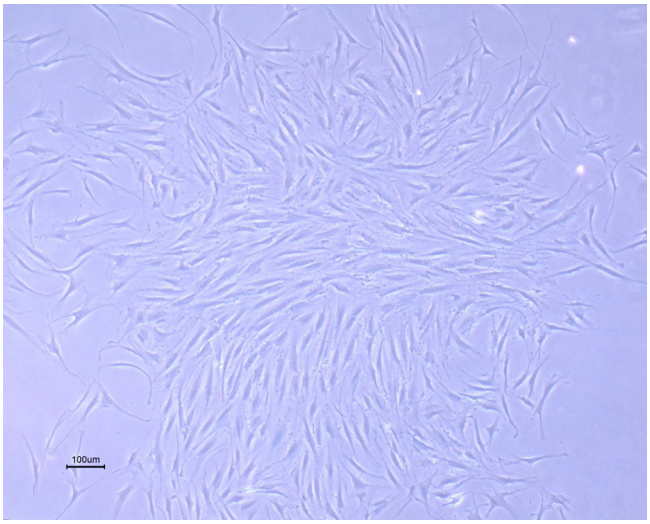

Figure 1 Morphology of bone marrow mesenchymal stem cells after culture for 1 week (inverted microscope).
Bone marrow mesenchymal stem cells exhibited a few thin and long processes. Scale bar: 100 μm.

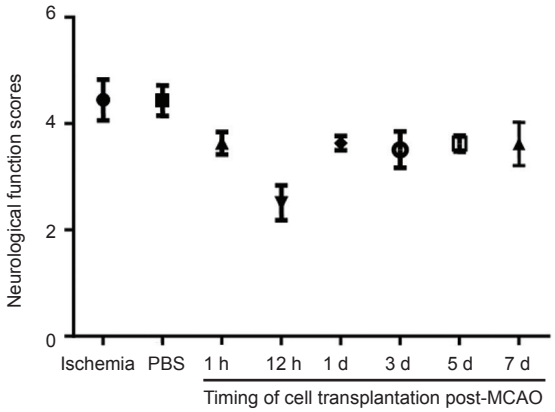

Figure 3 Recovery of rat neurological function following BMSC transplantation post-MCAO.
The neurological function scores in each group were assessed at 28 days after middle cerebral artery occlusion. Higher scores indicate more severe neurological deficits. The neurological function scores in the group that received bone marrow mesenchymal stem cell transplantation at 12 hours after middle cerebral artery occlusion were significantly lower than in the other groups ($P < 0.05$; one-way analysis of variance, $n = 15$). BMSC: Bone marrow mesenchymal stem cell; MCAO: middle cerebral artery occlusion; h: hour(s); d: day(s).

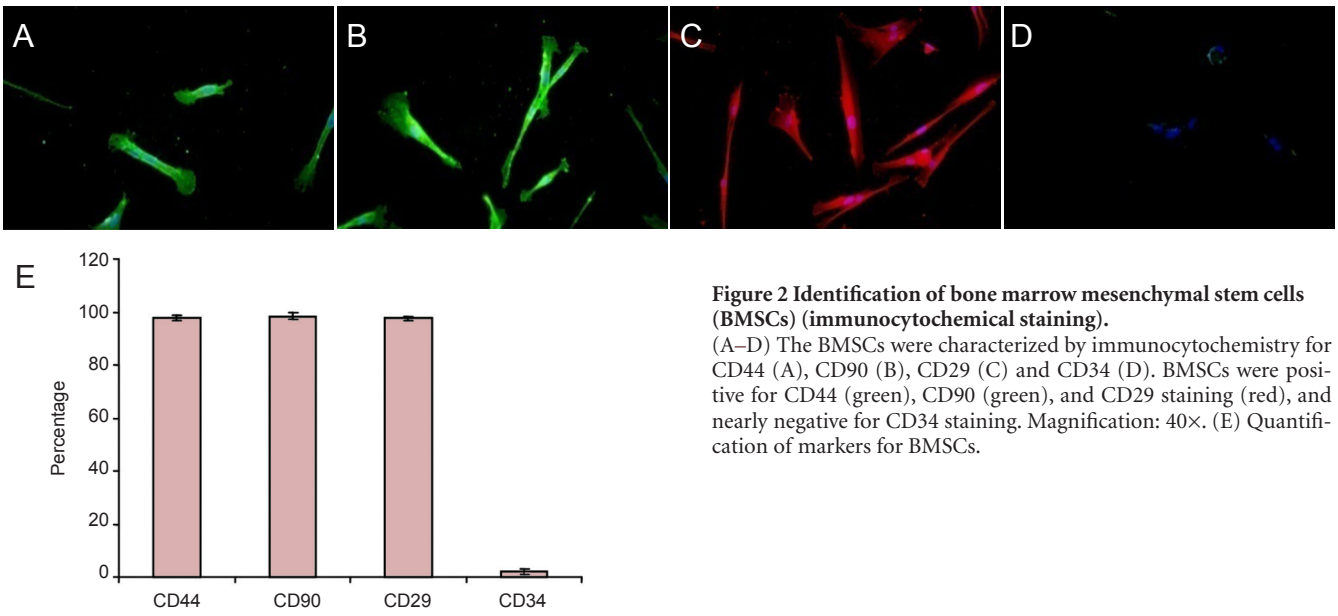

Figure 2 Identification of bone marrow mesenchymal stem cells (BMSCs) (immunocytochemical staining).
(A–D) The BMSCs were characterized by immunocytochemistry for CD44 (A), CD90 (B), CD29 (C) and CD34 (D). BMSCs were positive for CD44 (green), CD90 (green), and CD29 staining (red), and nearly negative for CD34 staining. Magnification: 40×. (E) Quantification of markers for BMSCs.

Neurological function evaluation

Rat neurological function evaluation was performed every 2 days within 28 days after MCAO. At 28 days after MCAO, the rats that underwent BMSC transplantation at 12 hours after MCAO had the lowest scores and best neurological outcomes (**Figure 3**).

Cell migration

The BMSC transplantation group (12 hours), *i.e.*, the group in which rats received BMSC transplantation at 12 hours after MCAO had more migrated cells than the other groups ($P < 0.05$) (**Figure 4**).

Cerebral infarct volume

Ischemic brain was determined by some ischemic signs including eosinophilic cytoplasm and pyknotic nuclei. Cerebral infarct volume in the BMSC transplantation (12 hours) group was significantly smaller than that in the other groups ($P < 0.05$; **Figure 5**).

Caspase-3 activity

Caspase-3 activity in ischemic cerebral tissue was evaluated through the use of spectrophotometer at 405 nm. Highest apoptosis rate appeared in the ischemia group and lowest apoptosis rate in the BMSC transplantation (12 hours) group.

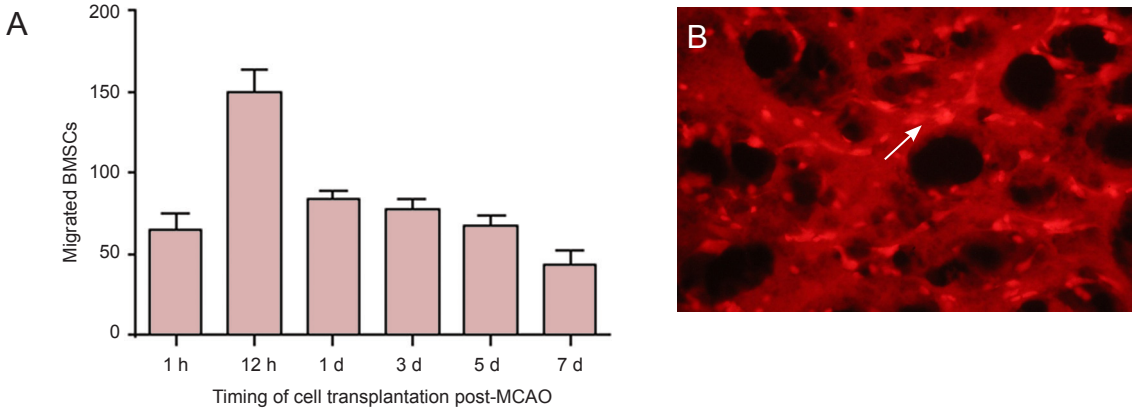

Figure 4 Distribution of bone marrow mesenchymal stem cells (BMSCs) injected at 12 hours after MCAO in the peri-infarct area.
The number of migrated BMSCs in the rats that received BMSC transplantation at 12 hours after MCAO was greater than that in the rats received cell transplantation at other time points ($P < 0.05$, one-way analysis of variance). (B) The migrated BMSCs (arrow), *i.e.*, PKH-labeled cells, in the peri-infarct area under the fluorescence microscope. MCAO: Middle cerebral artery occlusion; h: hour(s); d: day(s).

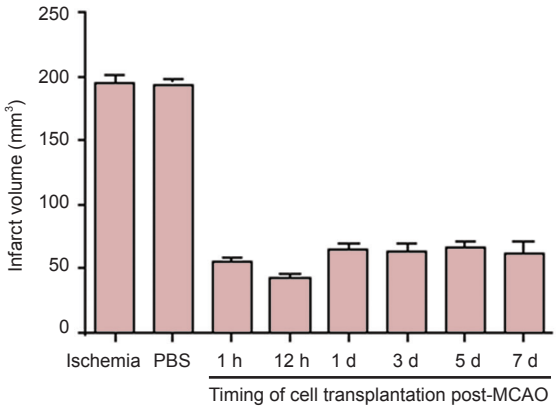

Figure 5 Comparison of cerebral infarct volume among different groups using hematoxylin-eosin staining.
Cerebral infarct volume in the cell transplantation (12 hours) group was smaller than in the other groups ($P < 0.05$). The data are expressed as the mean ± SD and were analyzed by the Kruskal-Wallis test. MCAO: Middle cerebral artery occlusion; h: hour(s); d: day(s).

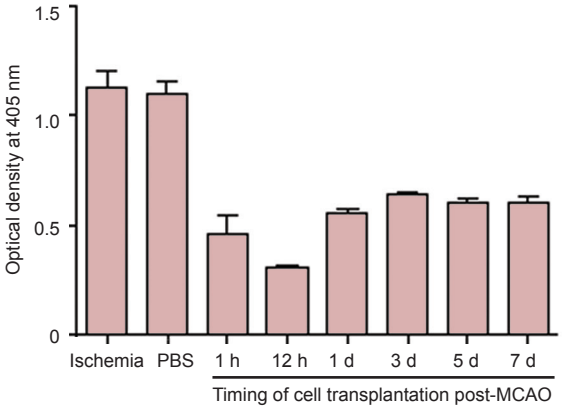

Figure 6 Comparison of caspase-3 activity in ischemic rat cerebral tissue among different groups.
Higher optical density indicates higher caspase-3 activity and higher incidence of apoptosis. The caspase-3 activity in the cell transplantation (12 hours) group was lower than in the other groups ($P < 0.05$). The data are expressed as the mean ± SD and were analyzed by one-way analysis of variance. MCAO: Middle cerebral artery occlusion; h: hour(s); d: day(s).

There was significant difference in the apoptosis rate between the BMSC transplantation (12 hours) group and the other groups ($P < 0.05$) (**Figure 6**).

Discussion

Results from this study showed that 12 hours after MCAO was the optimal time for BMSCs injection against cerebral ischemia. However, the underlying mechanism regarding this conclusion was not clarified in this study. In addition, injection of BMSCs reduced apoptosis and caspase-3 activity, which are consistent with previous findings (Chen et al, 2013). The present findings also showed that 12 hours after MCAO was also the most effective time for BMSC injection to diminish apoptosis.

There is evidence that MSC transplantation can improve neurological outcome and alleviate the symptoms of the disability (Li et al., 2002; Shen et al., 2007). It is also reported that the transplantation route of BMSCs influences the therapeutic effects on stroke (Cheng et al., 2015). Previous studies have shown that BMSC therapy exhibits neuroprotective effects on stroke by anti-inflammatory and anti-apoptotic effects of these cells, which were manifested as the significantly decreased expression of TNF-α, IL-1β mRNA and P-IκB-α, P-IKKβ, p53 and markedly increased expression of anti-apoptotic genes, such as IκB-α and Bcl-2 in the ischemic cerebral tissue (Okazaki et al., 2008; Gu et al., 2014).

In addition, BMSCs are capable of supporting nerve cells and increasing neurogenesis after cerebral ischemia by secreting some neurotrophic factors such as brain-derived growth factor (BDNF) and basic fibroblast growth factor (bFGF). Sygnecka et al. (2015) revealed that MSCs not only protected the viable cells against inflammation and secondary injury but also contribute to neural regeneration.

Presence of more migrated BMSCs in the peri-infarct area

indicates better effects of BMSC therapy on cerebral ischemia. More BMSCs injected at 12 hours after MCAO better reduce cell apoptosis (as caspase-3 activity shown). Some stem calls have the capacity to inhibit apoptosis by downregulating certain apoptotic genes such as *caspase-3* (Nasir et al., 2013) and reduce blood-brain barrier disruptions and endothelial damage (Ishizaka et al., 2013; Chung et al., 2015).

Caspases are the most important categories of cytokines that are involved in apoptosis; and caspase-3 is the activated death protease that catalyzes the specific cleavage of many important cellular proteins (Porter and Jänicke, 1999). Results from this study showed that BMSC transplantation reduced caspase-3 activity in the ischemic cerebral tissue. The lowest caspase-3 activity, indicative of the most effective anti-apoptotic effect, was acquired in rats receiving BMSC transplantation at 12 hours after MCAO. Therefore, BMSC transplantation after cerebral ischemia may help improve neurological function and reduce infarct volume and caspase-3 activity in affected individuals.

Collectively, 12 hours after ischemic stroke is the optimal time for BMSC transplantation against cerebral ischemia because this cell therapy can markedly diminish cerebral infarct volume, reduce apoptosis and caspase-3 activity, and thereby improve neurological function. BMSC therapy may be a new approach to help reduce the disability and morbidity of ischemic stroke patients.

Author contributions: *SMH designed and performed experiments, analyzed data and wrote the paper; MF and ZR performed experiments; SD developed analytical tools; and BS analyzed data. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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