

## Original Article

### The role of stem cells in the improvement of brain injuries after hypoxic ischemia

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#### Abstract:

**Introduction:** Nowadays, the important role of stem cells in treatment of many diseases such as stroke is well known. Stem cells derived from either bone marrow or cord bloods are good sources for tissues replacement after post embryonic injuries.

**Methods:** Fourteen-day-old Wistar rats were used in this study. Rats were subjected to internal carotid artery occlusion for 30 minutes. Then, animals were received intravenously  $2 \times 10^5$  Bromo Deoxy Uridine (BRDU) labeled- cord blood stem cells (CBSCs). Rats with hypoxic conditions that were not received any injection were assumed as a sham group. Intact animals who did not receive any injection or surgeries were used as a control group.

**Results:** Our results were evaluated according to behavioral tests and immunohistochemistry of the brain especially frontal cortex of the control, sham and experimental groups. Behavioral recovery was observed in the experimental group compared to the either the sham or the control group. In addition, histological studies demonstrated a reduction in ischemic cells in the experimental group compared to the sham group.

**Conclusion:** Intravenous transplantation can be a future line in treatment of infants with hypoxic who are exposed to irreversible damages.

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#### 1. Introduction

At this time, stroke is one of the most important causes of sensory-motor disability that can be due to occlusion of a capillary or ischemia. Hence, brain damaged cells cause irrecoverable complications. Although the administration of anti-thrombolytic drugs as well as complementary therapy such as rehabilitation and physiotherapy may improve signs after stroke, however there is not a definitive therapy for these neurological damages (1). Several studies were focused on the stem cell therapy for improvement tissue damages especially in the nervous system. Stem cell therapy had great potential to be considered as a treatment for stroke via transplantation therapy. Stem cells are undifferentiated cells that are capable becoming either their precursor cells or another cell type. Recently, stem cell research is rapidly increasing for therapeutic effects in brain disorders such as ischemic stroke, Parkinson

(2, 3, and 4), Huntington's, amyotrophic sclerosis, Alzheimer and multiple sclerosis (4, 5). It is hope that using stem cell will replace the damaged cells with new proliferated cells. Types of stem cells are isolated from different tissue sources that may be useful in treatment of brain ischemia. These cells include embryonic stem cells (6), adipose tissue, neural stem cells (7), stem cells derived from bone marrow and cord blood (8). Umbilical cord blood contains a population of stem cells that have the potential to differentiate into the neurons. Besides, these cells are less immunologic compared to the bone marrow-derived mesenchymal cells. So, the aim of this study was investigation effects of transplantation of cord blood-derived stem cells on motor recovery in the experimental models of ischemic stroke using behavioral test and immunohistochemistry.

## **2. Materials and Methods**

### **2.1. The isolation of mononuclear cells and labeled them with Bromo Deoxy Uridine (BRDU)**

After approval study by the Ethical Committee of Mashhad University of Medical Sciences, umbilical cord blood of mothers with range of 20- 40 years were collected in special bags containing dextrose adenine citrate phosphate. Women who had no history of smoking, alcohol or especial disease were selected from the Obstetrics and Gynecology Department of Ghaem Hospital, Mashhad, Iran. Blood samples were rapidly diluted 1:1 in phosphate buffer saline (PBS) and diluted again to a ratio of 8:3 in 15 ml centrifuge tubes with Ficoll-Paque centrifugation at 800 g for 20 min at room temperature. After that the upper phase was transferred into new tubes. After washing twice with PBS, samples were centrifuged at 800 g for 10 to 20 minutes. Collected cells were suspended at the bottom of the tube with 1 ml of serum. Then, the mononuclear cells were transferred into the culture flasks containing RPMI medium enriched with 10% bovine serum and antibiotics. Cells were labeled with 3 µg/ml BRDU and incubated at 37 °C for 24 hours. The viability rates of isolated mononuclear cells were assessed using a Neubaur haemocytometer and trypan blue dye.

### **2.2. Making an animal model of stroke or brain ischemia**

Twenty male Wistar rats (2 weeks old) were carried out in this study. Rats were anesthetized intra-peritoneally using 30 mg/kg ketamine and 4 mg/kg xylosine. Hypoxic ischemic model was created based on Hidetoshi's method. Briefly, after a midline cervical incision, right carotid artery was occluded using a string suture 6-0 for 30 minutes (10). Then, the skin incision was sutured and animals were kept under sterile conditions until recovery. Ten neonates were received intravenously  $2 \times 10^5$  stem cells at day 7 after the hypoxia - ischemia injury. Ten neonates with hypoxic conditions did not receive any stem cells and were considered as a sham group. Ten healthy pups without hypoxia were considered as a control group.

### **2.3. Investigation motor, behavioral changes**

Two behavioral tests were performed to evaluate motor disorders, motor coordination and somato-sensory defects in the rats. Behavioral tests were performed three times on days 1, 7 and 14 after stem cell injection as follows:

- According to De Ryck and coworker's method, function of each front and hind limbs on each side of the body was evaluated in 6 tests separately (11). For each test a score of zero (no response to movement of a limb), 1 (motor incomplete or delayed more than 2 seconds on a limb) and 2 (a fast moving and full body) was considered that sum of scores was 16 for a healthy animal.
- Based on Hua and coworkers (12), rats were abandoned in a corner and then count back from the left and right on 10 separate occasions and values were presented as a percent. Mouse back from both sides equally was normal and mouse want to turn to one side had a unilateral brain damage.

### **2.4. Investigation of histological changes**

All rats were anesthetized at day 14 after the last behavioral test and perfused with 100 ml cold saline followed by 100 ml Paraformaldehyde 4% in buffer. Then, their brains fixed in Paraformaldehyde fixative for 24 hours. The paraffin blocks were prepared from each brain and then were sectioned with six-micron thickness from blocks. Each 40th section of the histological series was stained with hematoxylin & eosin. Percent of the ischemic lesion in each section was calculated using image analysis system (Data Translation, Marlboro, MA). For tracing transplanted stem cell in the stratum, the damaged brain tissue was labeled with BRDU. The sections were reacted with anti-BrdU primary antibody and then were incubated with peroxidase-labeled secondary antibody. The sections were stained with DAB solution and evaluated using a light microscope.

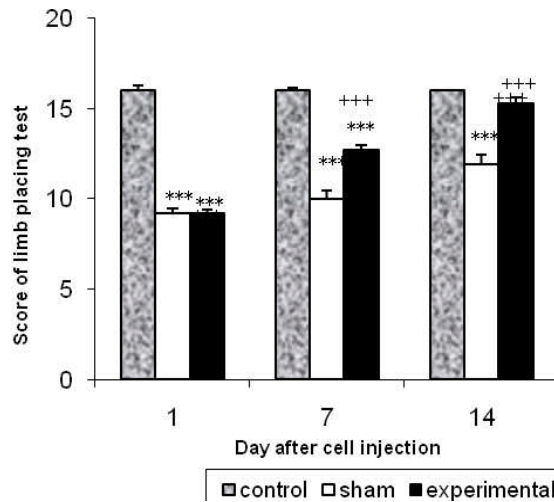
## 2.5. Statistical analysis

Data were analyzed using SPSS software and Duncan multiple range test. P values less than 0.05 were considered significant.

## 3. Results

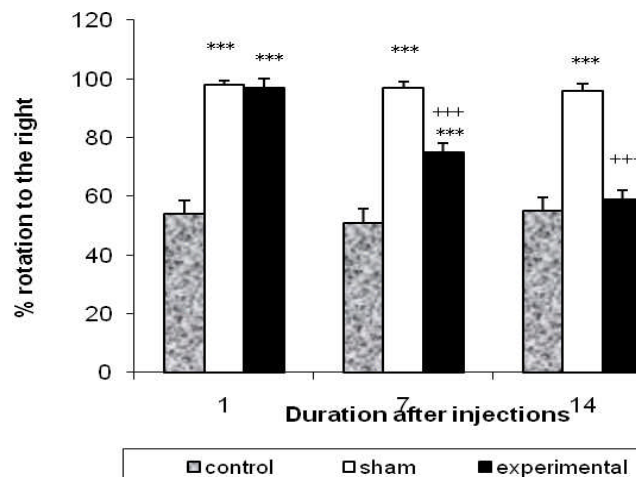
### 3.1. Improvement of the motor-behavior

The results of limb placing test showed that scores in the experimental group did not differ significantly compared to the sham animals on the first day after cell transplantation ( $9.2 \pm 0.25$  vs.  $9.2 \pm 0.2$ , respectively).



**Figure 1.** The effect of the injected stem cells derived from cord blood on Limb Placing Test score of ischemic stroke in rats. Animals in the experimental group were improved mobility in the limbs of the front and hind compared with the sham group on 14 days after injection ( $P < 0.05$ ).

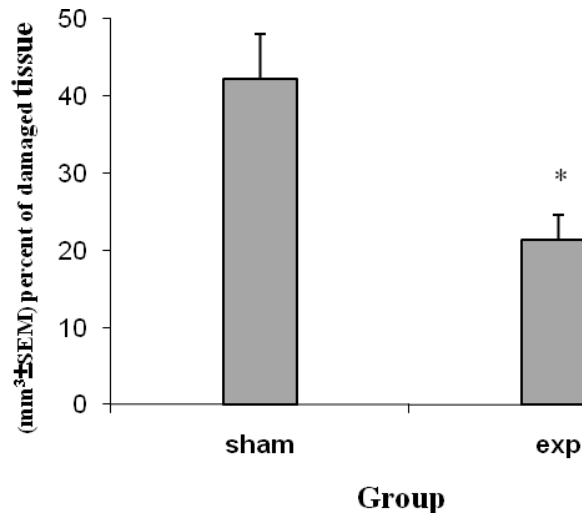
\*\*\* $P < 0.001$  vs control; +++ $P < 0.001$  vs Sham



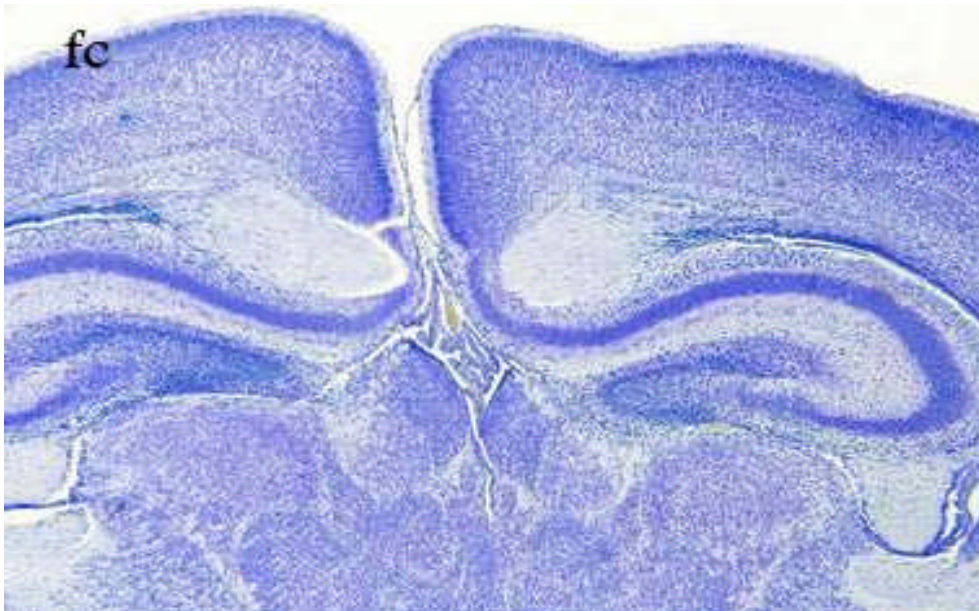
**Figure 2.** The effect of injected stem cells derived from the umbilical cord blood of rotation to the right on the Corner Turn Test in rats with ischemic stroke. Percent of turning to the right of the experimental group compared to the sham animals on 14 days after injection that shows a decreased level ( $P < 0.05$ )

\*\*\* $P < 0.001$  vs control; +++ $P < 0.001$  vs sham

The scores were then increased on the day 7 after injection in both groups. This increase was significantly higher in the experimental group than that of the sham group ( $12.7 \pm 0.26$  vs.  $10 \pm 0.47$ , respectively). These scores once more increased on the day 14 after cell injection ( $15.3 \pm 0.31$  vs.  $11.9 \pm 0.53$  for the experimental and sham groups, respectively). The control group's scores were 16 points in each three sessions. By considering the scores of the experimental and sham group, the control group had significant difference in the first and seventh days after injection. Although the difference with the sham group was continued until day 14 but no this difference was not significant (Figure1).



**Figure 3.** Percent of brain damaged tissue in comparison with healthy hemisphere in the experimental and sham groups at level less than 0.05



**Figure 4.** Coronal section of frontal cortex (fc) of 21-day rat brain hemisphere in the control group with low magnification (hematoxylin & eosine staining, scale bar 40  $\mu$ )

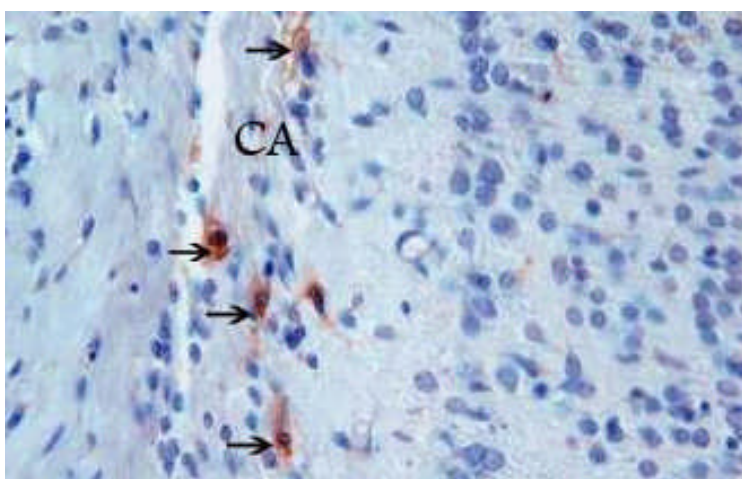
In Corner Turn Test, status of parity motor in hypoxic-ischemia rats were examined by the record of the numbers turns to the right and the values were presented as percent. Hence, this test was performed in the first, seven and 14 days after cell transplantation for all groups. There was no significant difference between scores of the experimental group ( $3 \pm 97$ ) compared to the sham group ( $1.3 \pm 98$ ) on day 1 after cell injection. The scores in the experimental group ( $3.7 \pm 75$ ) were significantly higher than that in the sham group ( $2.13 \pm 97$ ) on day 7 after injection and continued to be significant on day 14 after cells injection ( $3.14 \pm 0.59$  vs.  $2.21 \pm 0.96$ , respectively). Scores of the control group were  $4.52 \pm 54$  and  $4.82 \pm 51$ , respectively that were difference compared to the experimental group on 1 and 7 day after injection. The scores in the control group ( $4.53 \pm 53$ ) were significantly different compared to the experimental group on day 14 after injection (Figure 2).

### 3.2. Investigation the percent of the damaged brain tissue

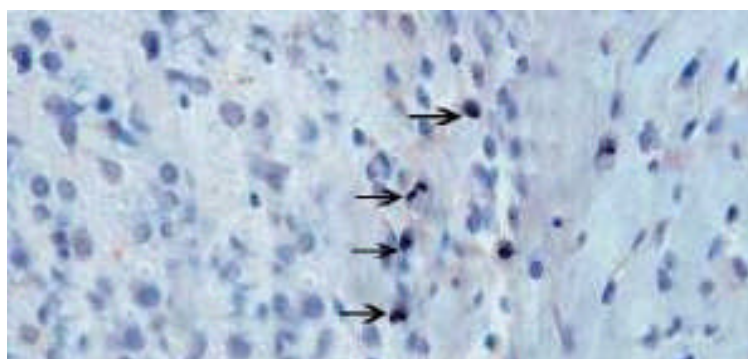
The histological studies demonstrated that percentage of the brain damaged tissue in the experimental group ( $21.4 \pm 3.2$ ) was significantly reduced compared to the sham group ( $42.2 \pm 5.73$ ) (Figure 3, 4).

### 3.3. The replacement of the labeled stem cells in the damaged area

The presence of injected stem cells into the experimental group was confirmed using immunohistochemistry for each group. The migration of labeled BRDU-stem cells were found in the light brown (Figure 5, 6). The damaged area of the brain is separable from purple color of the background. In the cortex of untreated hypoxic group observed both the choromatolysis phenomenon and cell death.



**Figure 5.** Section of frontal cortex of 21-days-old rat in the experimental group that arrows shows the labeled stem cells around capillary (CA) with brown color (scale bar=200  $\mu$ )



**Figure 6.** Section of the frontal cortex of 21-days-old rat in the sham group and arrows shows numbers of picnotic and dead cells.

#### 4. Discussions

Nowadays, many studies have focused on the therapeutic potential of the embryonic or adult stem cells in the repair of neurological damages. Using stem cells in the experimental models have been caused remarkable improvement in neurological disease (13). Stem cells that have investigated in these studies include: neural stem cells cultured from embryonic tissue, Neural stem/progenitor cells (NPCs) (15, 14), immortalization of neural cell lines (17, 16), endothelial and hematopoietic precursor cells isolated from bone marrow (19, 18), cord blood (21, 20) adipose tissue-derived stromal cells and peripheral blood (22, 23). Many studies have reported the benefit effects of intravenous transplantation of blood cord-derived stem cells into the brain. With regard to the therapeutic potential of the cord blood- derived stem cells, Chen and colleagues were investigated survive, differentiation and improvement of motor-neurotic activity in the hypoxic-ischemic rat models. Rats were received intravenously  $3 \times 10^5$  stem cells derived from human umbilical cord blood on 1 and 7 days after the surgery. Behavioral tests (Modified Neurological Severity Score and Rotard) as well as Immunohistochemistry staining was done for all groups. The results of the behavioral tests showed that injected cells can lead to increasing scores of experimental group at 24 hours after injury. The injected cell on 7 days caused significant changes only in points of Modified Neurological Severity Score test. Besides, Immunohistochemistry results showed the replacement destroyed cells with stem cells in the brain (24). Similarly, Willing & Partners in 2003 were created a laboratory model of ischemic stroke. Animals were injected cord blood- derived stem cells in both intravenous and direct delivery routes at 24 hours after injury. The results of motor-behavior tests of animals showed that both types of cell transplantation were effective in the improvement of the animal's spontaneous activity, but mobility recovery was observed only in the animals that were injected intravenously. Hence, they concluded that administration the cells via route acts better than direct injection into the damaged tissue (25). Injection different doses of the stem cells demonstrated that the motor-behavior recovery was dependent on the number of injected cells into the hypoxic models (26).

In our study was investigated the effects of intravenous transplantation of the stem cells derived from umbilical cord blood into laboratory models of hypoxia – ischemic. Our results demonstrated that intravenous injection of blood cord-derived stem cells causes improvement of motor-behavior in the treated groups compared to the untreated ones. Besides, histological studies confirmed that the labeled cells were present at the site of the damage. Behavioral improvements were evaluated using two behavioral tests. For the Modified limb placing test, the control animal's points was 16 while the scores of both the experimental and sham groups were significantly difference compared to the control group in the first and seventh days after the cell injection. However, there was no difference in the treated animals compared to the untreated ones on 14 day after injection. For second test, the rotation of the control normal animals to the right and left was 50% on 1 day after injection, while the experimental and sham groups tend to turn to the right near 100% on day 1 after injection. There was significant difference in the experimental and sham groups compared to the control group. There was no difference between the experimental and control groups on 14 days after injection and the percents of the rotation in the experimental animals was almost 50%.

#### 5. Conclusion

Our findings indicated that intravenous injection of the blood cord- derived stem cells led to behavioral improvement in the laboratory models. Besides, both a loss of damaged tissue in the hemisphere of the experimental group as well as replacement of the labeled cells in the damaged area confirmed that cord blood stem cells can be benefit for treatment of the hypoxia-ischemic stroke. However, this should be considered that a hypoxic- ischemia lesion may get involved other parts of the brain like motor cortex as well as the frontal cortex. Cells changes of the brain especially in the frontal cortex were evaluated in our study. So, lack of behavioral improvement may be relevant to other parts of the brain. Stem cells injected intravenously are benefit for treatment of stroke. We hope that intravenous transplantation be a treatment for infants with hypoxic who are exposed to irreversible damages. However, more studies are required for determination of the dose of injected cells and evaluation of effects in long period.

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