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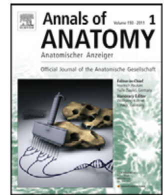

Research article

# Intravenous administration of human umbilical cord blood-mononuclear cells dose-dependently relieve neurologic deficits in rat intracerebral hemorrhage model

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SUMMARY

Human umbilical cord blood (HUCB) is now considered as a valuable source for stem cell-based therapies. Previous studies showed that intravascular injection of the HUCB significantly improves neurological functional recovery in a model of intracerebral hemorrhage (ICH). To extend these findings, we examined the behavioral recovery and injured volume in the presence of increasing doses of human umbilical cord blood derived mononuclear cells (HUC-MCs) after intracerebral hemorrhage in rats.

The experimental ICH was induced by intrastriatal administration of bacterial collagenase IV in adult rats. One day after the surgery, the rats were randomly divided into 4 groups to receive intravenously either BrdU positive human UC-MCs ($4 \times 10^6$, $8 \times 10^6$ and $16 \times 10^6$ cells in 1 ml saline, $n = 10$, respectively) as treated groups or the same amount of saline as lesion group ($n = 10$). There was also one group (control $n = 10$) that received only the vehicle solution of collagenase. The animals were evaluated for 14 days with modified limb placing and corner turn tests. The transplanted human UC-MCs were also detected by immunohistochemistry with labeling of BrdU.

Two weeks after infusion, there was a significant recovery in the behavioral performance when $4 \times 10^6$ or more UC-MCs were delivered ($P < 0.05$–$0.001$). Injured volume measurements disclosed an inverse relationship between UC-MCs dose and damage reaching significance at the higher UC-MCs doses. Moreover, human UC-MCs were localized by immunohistochemistry only in the injured area.

Intravenously transplanted UC-MCs can accelerate the neurological function recovery of ICH rat and diminish the striatum lesion size by demonstrating a dose relationship between them.
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## 1. Introduction

Intracerebral hemorrhage, which results from the spontaneous rupture of an intracranial vessel, is a subtype of stroke with high morbidity and mortality counting about 15% of all deaths from stroke and happens frequently as a major complication of thrombolytic therapy for acute ischemic stroke (Lapchak, 2002; Qureshi et al., 2001; Ribo and Grotta, 2006) and it leaves many survivors disabled (Broderick et al., 1999). The striatum component of the basal ganglia is the most common site for intracerebral hemorrhage and accounts for about one-third to one-half of all ICHs (Bogousslavsky et al., 1988; Foulkes et al., 1988; Mohr et al., 1978).

The prognosis of patients after ICH is poor, often much worse than that of patients with ischemic strokes of similar size. Currently, there is no available medical therapy for patients with ICH, and supportive care or invasive neurosurgical evacuation of hematoma in selective patients is all that can be done (Jeong et al., 2003).

Over the past years, cell transplantation has been proposed as a potential approach to the treatment of neurological disorders (Harris, 2008; Liao et al., 2009; Schouten et al., 2004). A number of different cell sources have been used successfully in various experimental models of stroke (Bliss et al., 2007). However, ethical considerations and limited availabilities restrict the application of several cell populations. One cell population of interest consists of human umbilical cord blood cells that are a source of hematopoietic stem/progenitor cells (HSCs) (Broxmeyer et al., 1989), mesenchymal stem/progenitor cells (MSCs) (Erices et al., 2000) and endothelial progenitors (Murohara et al., 2000). Since

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1989, when the first successful umbilical cord transplantation was reported in a child with Fanconi anemia, thousands of transplants have been carried out, as a therapy for certain malignant and nonmalignant hematological disorders (Madlambayan and Rogers, 2006). Moreover, in the last few years, preclinical studies have shown that human UC-MCs injected systemically in the acute phase of animal models of stroke have a therapeutic effect. These cells can reduce the area of brain infarction (Vendrame et al., 2004) and the inflammation (Vendrame et al., 2005), and increase the regenerative capacity of the brain (Taguchi et al., 2004), improving behavioral recovery (Chen et al., 2001). Additionally, HUC-MCs cells can secrete numerous neurotrophic factors (Sanberg et al., 2005). At the present time it is not known whether any of these stem/progenitor cells contribute to get over neurologic deficits seen in animals treated with human UCB. Since mononuclear cells acquired from human umbilical cord blood containing HSC are easy to obtain, ethically unproblematic and available for allogeneic approaches, they can be suitable candidates for cell therapies (Park et al., 2009).

Although several groups have reported beneficial effects of HUC-MCs administration after ischemic stroke, there has been a shortage of studies on the therapeutic benefit of HUC-MCs on hemorrhagic stroke and in more cell therapy studies that have been done in this brain disorder, researchers applied the stem cells to animals intracerebrally and only a few of them preferred to deliver the cells intravenously. Whereas, systematic delivery of these kinds of cells in experimental models can help to find better clinical approaches in the ICH patients. In this study, stem cells administrated intravenously and functional and histological recovery in ICH rats was evaluated. Additionally, because a specific dose range of this kind of cells has not been defined, the other aim of the present study was to answer this question: Which dose of intravenously delivered of HUCB-derived mononuclear cells could decrease injured volume of ICH in rats and leads to improvement of the neurological function. Since, acute rejection of transplanted cells typically occurs 14 days after transplantation (Larsson et al., 2001) and because HUCB cells did not activate cytotoxic CD8+ T cells, which would otherwise lead to acute rejection and during this time did not need to use an immunosuppressive agent for animals (Pan et al., 2005), in this study short term outcomes of human UCB-MCs transplantation was evaluated.

## 2. Materials and methods

### 2.1. Chemicals

Collagenase type IV, bromodeoxyuridine (BrdU) and antibodies were purchased from Sigma Chemical Co., USA and other materials provided from Merck Company.

### 2.2. Animal model of intracerebral hemorrhage

A total of 50 male Wistar rats, each weighing 250–300 g, were used throughout the study. All of them were housed in the same room under a constant temperature ($22 \pm 2\,^{\circ}$C) and illuminated 7:00 A.M. to 7:00 P.M., with food pellets and water available ad libitum. Animal handling and all related procedures were carried out in accordance with Mashhad University of Medical Sciences, Ethical Committee Acts.

The animals were anesthetized by 100 mg/kg/bw ketamine hydrochloride and 4 mg/kg/bw xylazine intraperitoneally (IP) and placed in a stereotaxic apparatus. A midline incision was made through the scalp to expose the skull. A 28-gauge needle was implanted into the striatum at coordinate's 0.2 mm posterior and 3.0 mm lateral to the bregma, depth 6.0 mm ventral to the surface of the skull (Del Bigio et al., 1996; Lee et al., 2006) and a 1.0 $\mu$l of sterile saline containing 0.24 U of bacterial collagenase IV was infused over a period of 5 min by a Hamilton syringe connected to needle into the striatum. Of course, for animals in the control group, only 1.0 $\mu$l of sterile saline was infused. At the end of infusion, the needle was slowly removed after an additional 5 min delay to prevent backflow and the wound was sutured. The rats recovered from surgery in a cage containing food and heated by an incandescent light bulb.

### 2.3. Collection and processing of umbilical cord blood

Samples of UCB were collected from full-term placenta of healthy women, nonsmokers, nondrinkers, age ranging from 20 to 40 years, in regard to ethnic group at the Obstetric Service of Hospital of the Ghaem hospital related to Mashhad University of Medical Sciences. Blood was collected in standard blood collection bags containing citrate phosphate dextrose adenine (CPDA) with a 20-gauge syringe. UCB samples were diluted at a proportion of 1:1 in a phosphate-buffered saline solution (PBS) without $Ca^{2+}$ and $Mg^{2+}$.

### 2.4. Isolation of UC-MCs by the standard density gradient technique and BrdU labeling

UCB samples were transferred to centrifuge tubes (15 ml) containing Ficoll–Paque solution (in a proportion of 8:3) and submitted to centrifugation at $800 \times g$ for 20 min in room temperature (RT) in order to isolate low-density umbilical cord blood mononuclear cells. The mononuclear cells cloudy interface layer (Buffy coat) was carefully removed by pipeting and transferred to a new tube and washed twice with PBS through centrifugation at $800 \times g$ for 10–20 min (Kawasaki-Oyama et al., 2008), then the cells were resuspended in 1 ml of separated serum of UCB and were counted and also their viability was enumerated by hemocytometer using Trypan blue dye exclusion method. Then, nucleated cells were seeded into each tissue culture flask in a RPMI medium supplemented by fetal bovine serum (10%) and 10 ml/l antibiotic. Then BrdU (3 $\mu$g/ml = 10 $\mu$M/L), labeled DNA during the S phase and currently has been used as a marker for in vivo and in vitro studies of isolated human stem cells for subsequent cell tracing within the recipient host (Chen et al., 2004; Lequeux et al., 2011; Seyfried et al., 2006) was added to cell suspension and cells then were incubated at 37 °C, humidified atmosphere containing 5% $CO_2$ for 24 h. After this incubation time, for assurance of being alive the UC-MCs, their viability and cell quantification were determined by the Trypan-blue method.

### 2.5. Flow cytometry

HUCB is a rich source of hematopoietic stem and progenitor cells (Pimentel-Coelho et al., 2010; Sirchia and Rebulla, 1999) and they can express cell surface markers of CD45 and CD34 (Hassanein et al., 2011). So specific monoclonal antibodies anti-CD45-FITC, anti-CD34-PE and the isotype control antibodies (immunoglobulin G1 [IgG1]) were used for the confirmation of the existence of these cells. Briefly, 100 $\mu$l of the samples containing $1 \times 10^6$ cell were incubated with 10 $\mu$l of each antibody for 20 min at RT in the dark. Erythrocyte lysis was performed by lysing solution of ammonium chloride.

Ten thousand cells were counted for each preparation on a FACS caliber fluorescence activated cell sorter (Beckton-Dickinson), and data analysis was performed with the WinMDI 2.8 software. The flow cytometer was properly aligned, and fluorochrome compensation for fluorescein isothiocyanate [FITC (FL1)] and phycoerythin ([PE (FL2)] was correctly tuned with respect to signal

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