

Umbilical cord-derived mesenchymal stem cell therapy for neurological disorders via inhibition of mitogen-activated protein kinase pathway-mediated apoptosis

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Abstract. The present study investigated the recovery and intrathecal administration of human umbilical cord-derived mesenchymal stem cells (HUC-MSCs) by lumbar puncture and analyzed the technical difficulties and short and long-term effects of UC-MSC transplantation in various neurological conditions. In total, 100 patients who underwent subarachnoid placement of UC-MSCs between December 2006 and May 2010 were included in the present study. The present study evaluated the number of attempts, localization of subarachnoid space and postprocedural complications. The Hauser Ambulation Index was employed for functional assessment. Clinical symptoms, the associated biochemical index and photographic examinations were observed regularly. HUC-MSCs were transplanted into mice as well as patients in order to determine the underlying therapeutic mechanisms. Technical difficulties were encountered in 31 patients (31%) in the form of general anesthesia supplementation and difficulty localizing lumbar space. Side effects were observed in 22 (22%) patients, which resolved with symptomatic treatment within 48 h. On follow-up one year later, functional indices improved in 47 (47%) patients. Transplantation of HUC-MSCs inhibited apoptosis and the protein expression of c-Jun N-terminal kinase and p38 as well as triggered the phosphorylation of P-42/44 extracellular-signal-regulated kinase. In conclusion, intrathecal administration of UC-MSCs is safe and effective with no long-term adverse effects in neurological disorders. HUC-MSCs may achieve these effects via the mitogen-activated protein kinase pathway. The results suggest that there is a promise of restoration of lost tissue and improvement

of function in patients with profound neurological defects. These data support expanded double blind, placebo-controlled studies for this treatment modality.

Introduction

Mesenchymal stem cells (MSCs) derived from the human umbilical cord (HUC), known as HUC-MSCs, represent a prospective cell source and hold great promise for tissue engineering and therapy (1,2). Compared with bone marrow MSCs they exhibit considerable advantages including the lack of ethical controversy, accessibility, extraction procedures that are painless for donors, a reduced risk of contamination, osteogenic differentiation capacities, *ex vivo* expansion, faster proliferation and a higher immunomodulatory capacity (2). Furthermore, there are few ethical restrictions or medico-legal limitations on extracting and applying these cells (2,3). The ample resources of the cords and the feasible cryopreservation of HUC-MSCs allow these cells to be preserved for engineering applications in the future (3). Animal experiments have demonstrated that HUC-MSCs may be useful in the treatment of neuron disease and multiple sclerosis (3-6). However, the safety and efficacy of stem cell therapy depends on the mode of cell administration. Furthermore, techniques aimed at increasing the number of HUC-MSCs isolated from umbilical cords (UCs) are extremely valuable.

Previous studies have indicated that the intrathecal treatment of UC-MSCs is safe, less invasive and a more convenient procedure involving no surgery (7-10). Currently, the efficiency of UC-MSC transplantation is limited to the grafting method (7-10). However, the grafting process may decrease the viability of the UC-MSCs. Therefore, enhancing the viability of UC-MSCs is the best way to improve the efficiency of UC-MSC transplantation.

The present study speculated that inhibiting the apoptosis of UC-MSCs may enhance the viability and survival of UC-MSCs. The role of the mitogen-activated protein kinase pathway in the pathogenesis of neurological disorders was also investigated. A prospective analysis was performed to assess the safety, therapeutic effect and the technical difficulties of HUC-MSCs intrathecal infusion in patients.

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Patients and methods

Characteristics of participants. In total, 100 patients with neurological disorders were recruited between December 2006 and May 2010 from The Second Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, Zhejiang, China). A total of 53 males and 47 females were enrolled in the present study with a male to female ratio of 1.12:1. The age at diagnosis ranged between 2 and 68 years (median 40 years). In terms of diagnosis, 30 patients had spinal cord injury, 15 patients had cerebral palsy, 15 patients had post-traumatic brain syndrome, 8 patients had post-brain infarction syndrome, 8 patients had spinocerebellar ataxias and 12 patients had motor neuron disease (Table I). The local institutional review board of The Second Affiliated Hospital of Medicine, under the auspices of the National Ministry of Health, approved application of the technique and written informed consent was obtained from each patient prior to initiation of the treatment. Patients were excluded from the present study if they met the following criteria: i) prior history of severe allergic reactions; ii) history of, or active, malignancy; iii) active systemic or severe focal infections, including HIV and syphilis; iv) active cardiac, pulmonary, renal, hepatic or gastrointestinal disease; v) coagulopathy or any other contraindication for lumbar puncture; vi) gastrostomy, tracheostomy or noninvasive ventilatory support as these can effect the prognosis and end-point measurements; vii) any severe psychiatric disorder and viii) any immunodeficiency disease or condition. As per protocol, the pre- and post-treatment study assessments included complete blood counts, routine urine tests, analysis of liver function, renal function, electrolytes, sero-enzymology, blood glucose, blood lipids, cellular and humoral immunity, routine cerebrospinal fluid (CSF) and biochemical markers (biochemistry analyzer and Epics-XL flow cytometer; Beckman Coulter, Inc. (Pasadena, CA, USA).

Characteristics of HUC-MSCs. The isolation, culture and expansion of the HUC-MSCs were performed as previously described by Gu *et al* (10). In brief, human umbilical cord (HUC) was obtained from the Gynecology Department at Renmin Hospital, Hubei University of Medicine (Shiyan, Hubei, China). Tissue collection was approved by the Ethics Committee of Renmin Hospital and informed consent was obtained from the newborns' parents. The selected tissue was sliced into 1-2 mm³ pieces and then incubated with 0.075% collagenase type II (Sigma, St. Louis, MO, USA) for 30 min and with 0.125% trypsin (Gibco-BRL, Grand Island, NY, USA) for 30 min. To obtain the cell suspensions, the treated tissue was passed through a 100 mm filter. The low glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) and 5% fetal bovine serum (FBS; HyClone, Logan, UT, USA), supplemented with 10 ng/ml epidermal growth factor (Sigma), 100 mg/ml streptomycin (Sigma), 10 ng/ml vascular endothelial growth factor (Sigma), 100 U/ml penicillin and 2 mmol/l glutamine (Gibco-BRL) were employed in the present study. For the cultural condition, the cells were cultured in an appropriate atmosphere at 37°C with 5% CO₂. Once the cell monolayer was formed, flow cytometric analysis was used for the detection of CD29, CD105, CD44

Table I. Clinical conditions of the investigated patients.

Condition	Number of patients
Number of patients	100
Spinal cord injury	35
Cerebral palsy	20
Post-traumatic brain syndrome	20
Post-brain infarction syndrome	9
Spinocerebellar ataxias	8
Motor neuron disease	8
Patients with technical difficulties	31 (31.0%)
General anesthesia supplementation	18
Taylor's approach	7
Multiple attempts	6
Side effects	22 (22.0%)
Headache	13
Low-grade fever	5
Low back pain	2
Lower limb pain	2
Improvement in functional indices	47 (47.0%)
Spinal cord injury	16
Cerebral palsy	12
Post-traumatic brain syndrome	10
Post-brain infarction syndrome	6
Spinocerebellar ataxias	2
Motor neuron disease	1

and CD166 positive cells and for CD34, CD14, CD45, CD38 and HLA-DR negative cells.

Cell administration. Patients' computed tomography scans and/or magnetic resonance images of the spine and brain were reviewed prior to the procedure, and the intervertebral space between lumbar vertebrae three and four was selected for HUC-MSCs placement. HUC-MSCs were administered via intrathecal injection by lumbar puncture. Each patient received cell transplantation four to six times depending on the patient's condition, within an interval of 5-7 days. CSF (2 ml) was removed and replaced by 2 ml of cell suspension during the intrathecal injection. The needle was maintained in the same position for 5 min prior to withdrawal. Pediatric and uncooperative patients were administered general anesthesia prior to performing lumbar puncture. The present study evaluated the number of attempts, localization of subarachnoid space and postprocedural complications. All patients were monitored in the wards for 24 h and hydrated with 3 liters of fluid; ambulation was allowed 8 h postprocedure. Short and long term functional evaluation was performed using the Hauser Ambulation Index (HAI) by the HUC-MSCs transplant team on a regular basis. The HUC-MSCs transplant process was performed according to the study by Dalous *et al* (12).

Therapeutic effect. To the best of our knowledge there are no published criteria to measure therapeutic efficacy in the treat-

Table II. Spinal cord injury clinical profile.

Condition	Number
Number of patients	38
Pattern of injury	12
Complete transection	22%
Nontransection	78%
Injury to treatment duration	4 months to 2 years
Clinical and functional benefit	
<1 year	13 (35.3%)
>1 year	6 (18.7%)
Pattern of motor improvement according to HAI ^a	
<1 year	13
Posttransplant grade	
4	2
5	3
6	2
7	3
>1 year	5
Posttransplant grade	
6	3
7	1
8	1
Autonomic improvement	12
<1 year	8
Catheter free	2
Intermittent catheterization	3
Bowel sensations and sweating	2
>1 year	4
Intermittent catheterization	3
Improved bladder tone	1

^aFifteen patients were HAI grade 9 prior to transplantation. HAI, Hauser Ambulation Index.

ment of HUC-MSCs for neurological disorders. The HAI was applied for the evaluation of treatment efficacy (11). The HAI is a rating scale developed by Hauser *et al* (11) to assess mobility by evaluating the time and degree of assistance required to walk 25 feet. The scores ranged between 0 (asymptomatic and fully active) and 10 (bedridden) as follows: 0 = asymptomatic, fully active; 1 = walks normally, however, reports fatigue that interferes with athletic or other demanding activities; 2 = abnormal gait or episodic imbalance, gait disorder is noticed by family and friends, able to walk 25 feet (8 meters) in ≤ 10 sec; 3 = walks independently, able to walk 25 feet in ≤ 20 sec; 4 = requires unilateral support (cane or single crutch) to walk, walks 25 feet in ≤ 20 sec; 5 = requires bilateral support (canes, crutches or walker) and walks 25 feet in ≤ 25 sec or requires unilateral support but requires >20 sec to walk 25 feet; 6 = requires bilateral support and >20 sec to walk 25 feet, may use wheelchair on occasion; 7 = walking limited to several steps with bilateral support, unable to walk 25 feet, may use wheelchair for the majority of activities; 8 = restricted to wheelchair, able to transfer self independently; 9 = restricted to wheelchair, unable to transfer self independently; 10 = bedridden.

Results

Autonomic improvement of spinal cord injury. Administration of HUC-MSCs via intrathecal routes was well tolerated during the clinical treatment course. Out of 88 patients, technical difficulties were encountered in 20 patients (23%), 12 of which required general anesthesia supplementation, three required Taylor's approach and five required multiple attempts for the localization of subarachnoid space. In total, 10 patients suffered from postprocedural headache, which was relieved within 24 h with analgesics, hydration and rest; 3 patients had low-grade fever lasting for 24 h; 3 patients had lower back pain and 2 patients had lower limb pain, which was responded to within 24 h of symptomatic treatment. On long-term follow-up, functional indices improved in 50 (31.67%) patients, including 15 patients with spinal cord injury, 10 with cerebral palsy, 10 with post-traumatic brain syndrome, 5 with post-brain infarction syndrome, 5 with spinocerebellar ataxias and 5 with motor neuron disease. Patients with cerebral palsy and post-traumatic brain syndrome demonstrated improvement in muscle tone, rigidity and spasm (Table I).

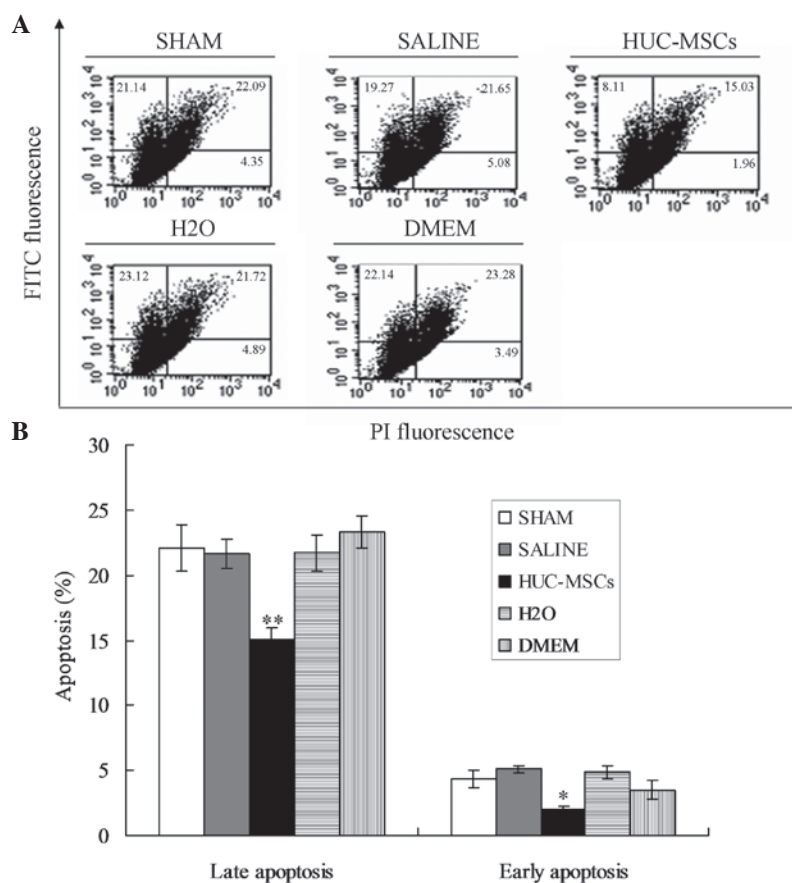


Figure 1. Apoptosis was observed in UC-MSC-treated mice. (A) Annexin V/PI double staining assays of cells. (B) Statistical analysis. The X axis indicates the number of PI stained cells. The Y axis indicates the number of Annexin V-FITC stained cells. The percentage of the cells labeled with only Annexin V (counted in Q1 region) in each preparation are shown in each figure. The results from three independent experiments are shown. The UC-MSC-treated group against the SHAM group are presented as the mean \pm standard deviation and illustrated as * $P < 0.05$ and ** $P < 0.01$, respectively. HUC-MSCs, human umbilical cord mesenchymal stem cells; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; PI, propidium iodide.

Out of the 30 spinal cord injury patients, 20 had a previous history of spinal surgery. In 15 spinal cord injury patients with HAI scale improvement, 10 patients had an injury period of <1 year, whereas 5 had an injury period of >1 year. A total of 15 patients showed improvement in motor power (10 with an injury period of <1 year and 5 >1 year). Prior to treatment, all these patients were HAI grade 9. Following HUC-MSC transplantation, one was grade 4, two were grade 5, three were grade 6 and four were grade 7. A marked improvement was observed in four bedridden patients who were able to walk with the help of a walker (HAI grade 9-HAI grade 4/5). In 12 patients, autonomic improvement was observed, including 8 patients with an injury period of <1 year. Of these, three became catheter-free and two required intermittent catheterization. In addition, 3 patients showed improvement in bowel sensations and sweating. A mixed motor and autonomic improvement was observed in 8 of 30 patients (Table II).

HUC-MSC transplantation inhibits apoptosis. In order to investigate the therapeutic effect of HUC-MSCs on neurological disorders, HUC-MSCs were transplanted into mice. The results indicated that HUC-MSCs were able to significantly inhibit apoptosis of neurocytes compared with the other groups (Fig. 1; $P < 0.01$).

HUC-MSCs inhibit apoptosis via the mitogen-activated protein kinase (MAPK) and extracellular-signal-regulated kinase (ERK)1/2 pathways. The activation of the other two members of MAPKs, c-Jun N-terminal kinase and p38, decreased the rate of apoptosis following treatment with HUC-MSCs (Fig. 2). On the basis of these observations, the effect of normothermic ischemia-reperfusion stress on the different members of the MAPK family were investigated. The expression of phospho-p 44/42 ERK1/2 was significantly higher in the liver of animals treated with HUC-MSCs (Fig. 3).

Discussion

Mesenchymal stem cells (MSCs) are highly proliferative *in vitro*, have multilineage differentiation potential and are able to be obtained from various tissue sources, including the bone marrow (BM), adipose tissue and umbilical cord blood. The BM is the main source for isolation of MSCs and BM-derived MSCs (BM-MSCs) have been studied extensively. However, BM harvesting is a highly invasive method for the donors. Furthermore, the multipotent differentiation potential, maximal lifespan of BM-MSCs and proliferation efficiency are correlated with the age of the patients. In addition, MSCs can be isolated from umbilical cord Wharton's jelly and differentiate into different types of cells, including adipocytes, osteocytes,

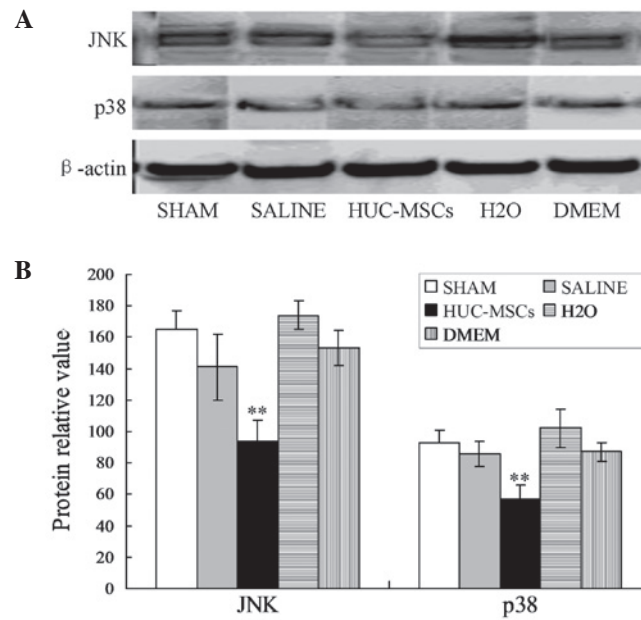


Figure 2. HUC-MSC transplantation results in a decrease of JNK and p38 in mice. (A) Western blot analysis; (B) statistical analysis. The UC-MSC-treated group compared with the SHAM group are presented as the mean \pm standard deviation and illustrated as **P<0.01, respectively. HUC-MSCs, human umbilical cord mesenchymal stem cells; DMEM, Dulbecco's modified Eagle's medium; JNK, c-Jun N-terminal kinase.

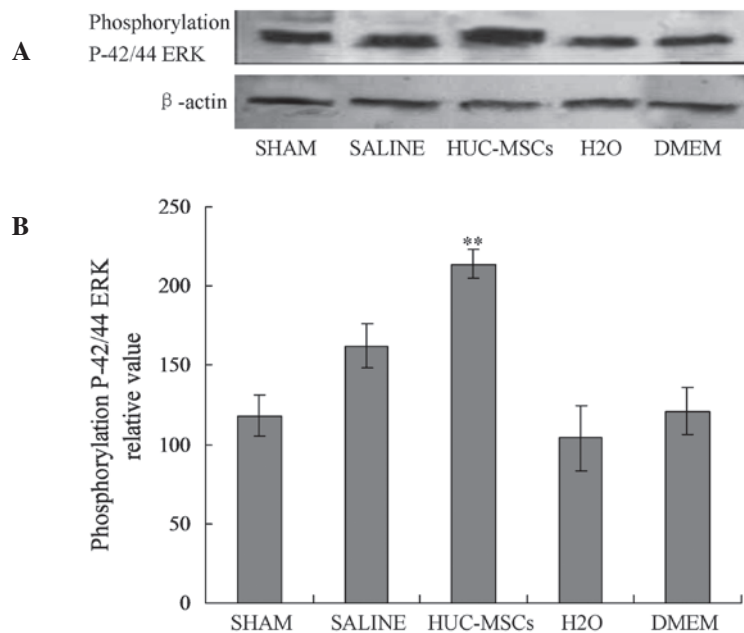


Figure 3. P-42/44 ERK phosphorylation may be involved in the inhibition of apoptosis in UC-MSC-transplanted mice. (A) Western blot analysis; (B) statistical analysis. The UC-MSC-treated group compared with the SHAM group are presented as the mean \pm standard deviation and illustrated as **P<0.01, respectively. UC-MSCs, umbilical cord mesenchymal stem cells; ERK, extracellular-signal-regulated kinase; DMEM, Dulbecco's modified Eagle's medium.

chondrocytes and neurons. Compared with the MSCs isolated from other tissues, UC-MSCs can differentiate into many types of cell, including blood, nerve and bone cells. Furthermore, this type of MSC does not express the major histocompatibility complex (MHC) class II (HLA-DR) antigens (13), which is expressed in other types of MSCs. Several studies have indicated that UC-MSCs retain their viable status, and are not rejected even four months following transplantation. Therefore, immune suppression is unnecessary for UC-MSCs transplantation, which suggests that UC-MSCs are a feasible and stable cell source for transplantation. Notably, the umbilical cord can be

easily obtained and isolated. Thus, UC-MSCs transplantation may act as a potential MSC cell source for tissue engineering.

Several studies have demonstrated that the transplantation of UC-MSCs is a potential therapeutic strategy for the treatment of central nervous system diseases, including cerebrovascular disease, nerve degenerative diseases, spinal cord injury and cerebral palsy. The transplantation path in cell therapy is diverse (14-16) and mainly includes the intervention path, local implantation, intravenous route and lumbar puncture way. Migration toward pathology is the first critical step in UC-MSCs engagement during regeneration and it is hypothesized that the

inflammatory response itself guides the behavior of potentially reparative UC-MSCs. It has been revealed that introducing UC-MSCs into the subarachnoid space of the spinal cord transports the cells through CSF and allows more efficient delivery of cells to the injured area of the central nervous system compared with the intravenous route.

Intrathecal administration of UC-MSCs is safe, less invasive and a more convenient procedure involving no surgery. However, the efficacy of UC-MSCs transplantation is limited by the grafting method. The mechanical process of grafting may decrease the viability and survival of the transplanted MSCs. Therefore, for the transplantation of MSCs it is necessary to improve the viability and survival of UC-MSCs prior to transplantation (14). The present study examined the viability of MSCs using the trypan blue exclusion assay. The patient should be positioned to ensure the intervertebral spaces are detectable for lumbar puncture for transplantation of cells into the CSF.

At present, the optimized method for injecting cells is to slowly deliver a fixed volume at a constant rate. The quantity and density of transplanted MSCs are critical to ensure an adequate number of cells for grafting and optimal survival. For the injection procedure, the needle tip should be left in the same position after the cells have been injected.

Notably, UC-MSCs cell transplantation was able to inhibit apoptosis (Fig.). It is well established that cell apoptosis can trigger neurological diseases and degenerative diseases. In the present study, UC-MSCs improved the disease status through inhibiting the apoptotic process of the cells.

In the present study, a significant number of patients with spinal cord injury were included. A lumbar puncture was performed in these patients, however, this was technically difficult (17-20) due to the following reasons: pathological scoliosis and positional difficulties. Certain other factors, including cognitive, behavioral and communication problems as well as coexisting diseases and the specific drug therapy methods, affected the anesthetic management. In addition, other problems affected the transplantation of MSCs, including gastroesophageal reflux, electrolyte imbalance and pulmonary aspiration in the present study. Furthermore, the positioning of the patients for the transplantation was difficult as analgesia and inadequate anesthesia may lead to increased muscle tone and spasm. Thus, judicious use of an anesthetic agent intraoperatively was critical to ensure a relaxed peri-operative and post-operative period. Following intraspinal injection of UC-MSCs in this patient population, certain patients (n=13) suffered from postprocedural headache. This was possibly due to numerous reasons, including the alteration in CSF circulation, leakage of CSF and the use of a large-bore spinal needle, which was essential to prevent any mechanical damage to the cells during infusion. Furthermore, 3 patients suffered from lower back pain and 2 patients had lower limb pain, possibly due to nerve root injuries; however, they recovered quickly. All the side effects resolved within 24 h with no long-term sequelae.

In conclusion, the present study indicated that subarachnoid transplantation with UC-MSCs in neurological disorders may be a promising therapy, which is relatively safe, simple to perform and has no long-term adverse effects. However, certain studies have provided different conclusions, and thus this method requires further investigation so that the potential of this therapy may be fully realized.

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