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Original Article

Therapeutic effects of human umbilical cord mesenchymal stem cells transplantation on hypoxic ischemic encephalopathy

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Abstract: Objective: Human umbilical cord mesenchymal stem cells (hUC-MSCs) hold substantial promise for the treatment of ischemic neurological disease, but few clinical data are currently available about its therapeutic effects in hypoxic ischemic encephalopathy (HIE). This study is to evaluate the effects of hUC-MSCs transplantation on patients with HIE. Methods A total 22 patients with HIEwere randomly divided into hUC-MSCs transplantation group (n = 12) and control group (n = 10). After isolation, hUC-MSCs were cultured for 3 to 5 passages in vitro and then intravenously administered to HIE patients in the transplantation group, while the control group received routine treatment only. The outcomes of HIE patients were evaluated at designated time points by clinical assessment scales, including NIHSS, Barthel Index, MMSE, $HAMA_{24}$, $HAMD_{14}$ and UPDRS. Results: hUC-MSCs were identified by morphological analysis and flow cytometry assays before clinic transplantation. No significant differences of demographic characteristics were observed between the two groups of subjects. Compared to the control group, hUC-MSCs transplantation markedly improved the outcomes of HIE patients leading to better recovery of neurological function, cognition ability, emotional reaction and extrapyramidal function. No significant adverse effects were found in subjects with hUC-MSCs transplantation during a 180-day follow-up period. Conclusion: These data suggest that hUC-MSCs therapy markedly improves the outcomes of patients with HIE, which is potential for the routine treatment of ischemic neurological disease.

Keywords: Human umbilical cord mesenchymal stem cells, transplantation, hypoxic-ischemic encephalopathy

Introduction

Hypoxic ischemic encephalopathy (HIE) is a clinical condition that occurs when the entire brain is partially or completely deprived of oxygen supply due to lack of cerebral blood flow. The most common cause of HIE in adult subjects is stroke, although it is also induced by traumatic hemorrhagia, cardiopulmonary resuscitation, carbon monoxide poisoning and so on. Thrombolytic therapy represents a significant advance in acute HIE, which however is necessary to be performed within the early hours of ischemia [1]. Since many patients suffered from acute HIE are not able to arrive at hospital in time, acute HIE remains the major cause of long-term disability in the industrialized world [2]. Although significant advances have been achieved in supportive care, effective therapies for ischemic encephalopathy remain lacking in clinic. It is of great interest to develop novel treatments for HIE that could be administered in a later time window after acute ischemia injury.

An emerging therapeutic approach for organ ischemia injury is the use of mesenchymal stromal cells (MSCs). These nonhematopoietic multipotent cells are able to down-regulate immune responses and promote tissue healing after ischemia/reperfusion injury [3]. It has been revealed that Human MSCs are able to inhibit

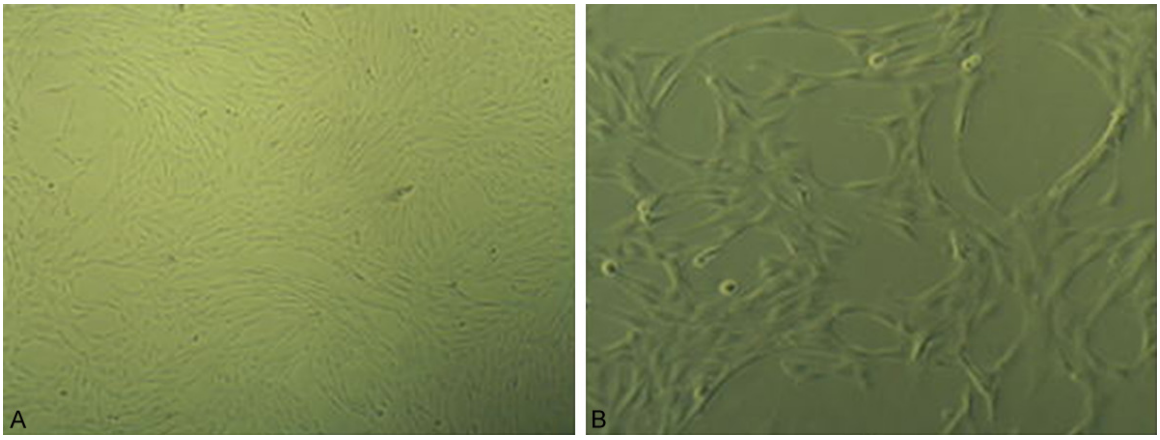

Figure 1. Microscopic images of cultured hUC-MSCs. Cell morphology of hUC-MSCs at passages 4 was observed under microscope. Magnification = (A) 100 ×, (B) 200 ×.

activation of diverse inflammatory cells, down-regulate expression of various chemokines and promote the generation of immune cells [4]. However, autologous MSCs are not immediately available upon request because isolation and expansion to sufficient numbers of cells requires weeks, resulting in treatment delay in emergency conditions, like stroke. Although allogeneic bone marrow derived MSCs from healthy donors provide an alternative for future cell therapy, the collection and isolation of human MSCs from donor bone marrow require invasive and often undesirable procurement procedures.

Recent studies have indicated that human umbilical cord is a promising source of mesenchymal stem cells (hUC-MSCs) [5]. hUC-MSCs can be isolated from umbilical cord blood [6], umbilical vein subendothelium and Wharton's jelly [7], which are characteristic of self-renewing and expression of specific cell surface markers of MSCs. Unlike bone marrow stem cells, hUC-MSCs have a painless collection procedure and faster self-renewal properties [8]. In addition to the ease of their collection, storage and transport, hUC-MSCs have many other advantages such as the wide range of sources and little ethical controversy. It is known that hUC-MSCs, under different circumstances in vitro, can directly differentiate into various neural cell lineages, including neurons, oligodendrocytes, astrocytes, and microglial cells [9, 10]. These multilineage differentiation potentials of hUC-MSCs hold tremendous interest of their application in neurological regeneration and repair [11]. Importantly, hUC-MSCs are also well tolerated by the host immune system after transplantation and thus can exhibit a powerful immunosuppressive activity, which have been consistently shown valuable for immune regulation in autoimmune neurologic disorders [12-14].

Recently, transplantation of hUC-MSCs has been successfully applied to patients with sequelae of traumatic brain injury, resulting in remarkable improvement on neurological functions [15]. However, the therapeutic effects of hUC-MSCs on HIE patients remains uncertain due to lack of clinical investigations. In this study, we aimed to examine the safety and efficacy of hUC-MSC transfusion in HIE patients. These findings could support the therapeutic potentials of hUC-MSCs in treatment of ischemic degenerative diseases.

Results

Characterization of hUC-MSCs

hUC-MSCs were successfully isolated from human umbilical cord and cultured for 3-5 passages before intravenous transplantation. Cultured hUC-MSCs were characteristic of fibroblast-like fusiform morphology on the flask (**Figure 1**). Flow cytometry demonstrated that hUC-MSCs expressed high levels of typical MSCs markers, including CD73 and CD44. In contrast, the expression levels of hematopoietic cell markers, including CD45 and CD34, were very low in these cells. The absence of the major histocompatibility complex II marker,

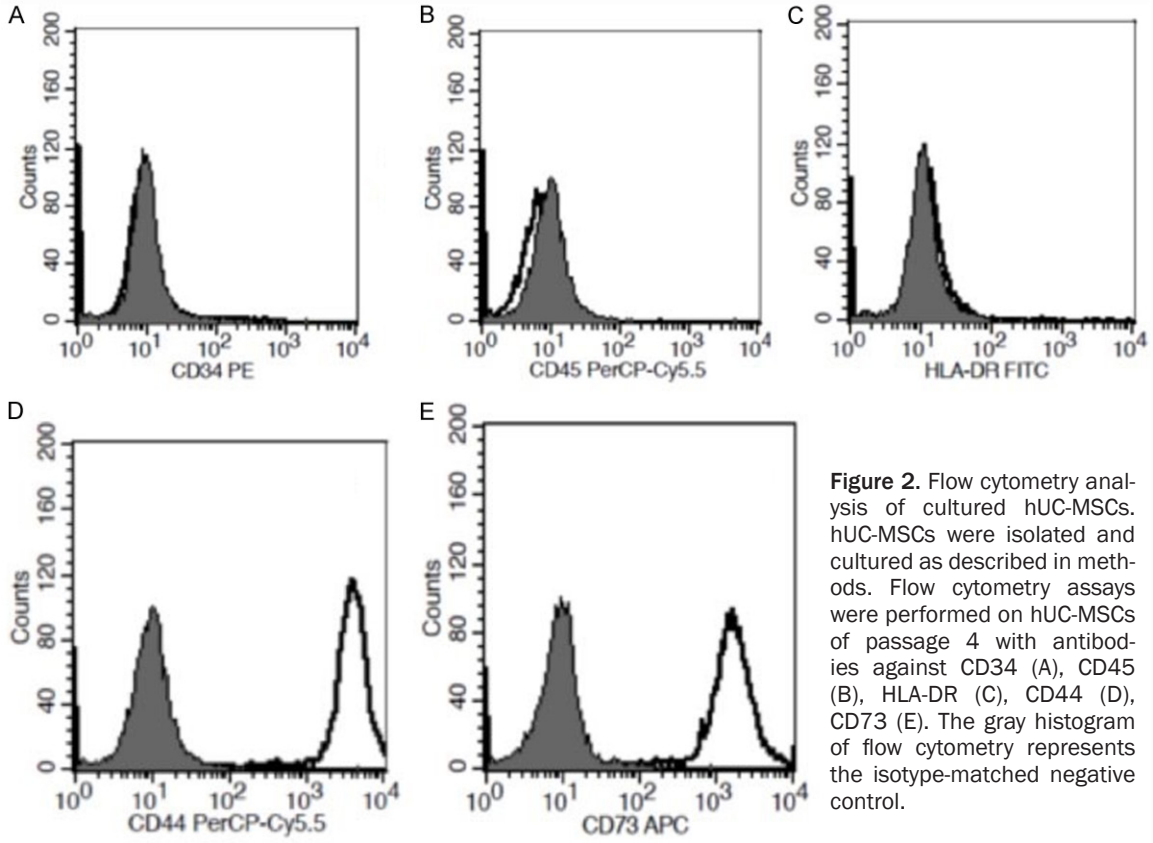

Figure 2. Flow cytometry analysis of cultured hUC-MSCs. hUC-MSCs were isolated and cultured as described in methods. Flow cytometry assays were performed on hUC-MSCs of passage 4 with antibodies against CD34 (A), CD45 (B), HLA-DR (C), CD44 (D), CD73 (E). The gray histogram of flow cytometry represents the isotype-matched negative control.

Table 1. Demographic characteristics of patients with HIE

Variables	Control group n = 10	Transplantation group n = 12	*P*
Gender, n (%)			1.000
Male	6 (60.00)	8 (66.67)	
Female	4 (40.00)	4 (33.33)	
Age (year), mean ± sd.	63.3±6.11	58±7.4	0.098
Min	51	45	
Max	71	68	

HLA-DR, was also consistent with the typical feature of hUC-MSCs (**Figure 2**).

Baseline patient characteristics

The baseline characteristics of all 22 enrolled subjects were described in **Table 1**. No statistical difference of demographics was observed in age and gender between the two groups, indicating successful randomization.

Evaluation on functional recovery

NIH Stroke Scale (NIHSS) assessment was applied to evaluate the outcomes of neurologic deficit after HIE (**Table 2**). No difference in NIHSS was observed between the two groups before the application of hUC-MSCs treatment (P = 0.933). Slow recovery of brain functions was observed in control subjects while comparing the NIHSS at day 0 (14.3±4.35) with day 180 (7.36±3.64). hUC-MSCs transplantation markedly accelerated the functional recovery of injured brained and improved the outcomes of HIE as indicated by significant decline of NIHSS from day 0 (14.08±6.93) to day 180 (334±3.79). As indicated by score difference, neurologic improvement in the transplantation patients was better than controls and increased with time, suggesting long-term beneficial effects of stem cell transplantation on HIE.

In line with NIHSS assessment, data of Barthel Index suggested HIE patients in transplantation group achieved better performance in activities of daily living than controls at all designated time points (**Table 3**). The score differences between two groups were respectively significant at day 14 (P = 0.011), day 90 (P = 0.025) and day 180 (P = 0.060), further sup-

Table 2. NIHSS score of patients with HIE pre- and post-transplantation (mean ± sd.)

Time	Variables	Control group n = 10	Transplantation group n = 12	*P**
0 day	NIHSS score	14.3±4.35	14.08±6.93	0.933
14 days	NIHSS score	11.8±4.02	8.91±4.06	0.314
	Difference	-2.5±0.53	-4.09±2.66	0.152
90 days	NIHSS score	10.9±3.87	7.91±4.01	0.296
	Difference	-3.4±0.97	-5.09±2.88	0.090
180 days	NIHSS score	7.36±3.64	3.34±3.79	0.248
	Difference	-3.6±1.96	-5.64±3.01	0.058

Difference = NIHSS score (Days after treatment - Day 0); *comparison between control and transplantation group.

Table 3. Barthel Index of patients with HIE pre- and post-transplantation (mean ± sd.)

Time	Variables	Control group n = 10	Transplantation group n = 12	*P**
0 day	Barthel Index	44.5±15.36	43.18±17.65	0.887
14 days	Barthel Index	51±15.78	56.82±16.17	0.752
	Difference	6.5±5.3	13.64±5.52	0.011
90 days	Barthel Index	52.5±14.77	59.09±19.85	0.722
	Difference	8±5.87	15.91±8.61	0.025
180 days	Barthel Index	53.5±13.13	59.55±20.67	0.747
	Difference	9±6.15	16.36±10.02	0.060

Difference = Barthel Index (Days after treatment - Day 0); *comparison between control and transplantation group.

Table 4. MMSE score of patients with HIE pre- and post-transplantation (mean ± sd.)

Time	Variables	Control group n = 10	Transplantation group n = 12	*P**
0 day	MMSE score	19.7±5.54	18.17±10.04	0.895
14 days	MMSE score	20.3±5.56	23.17±5.91	0.823
	Difference	0.6±1.78	5±5.17	0.015
90 days	MMSE score	20.5±5.84	23±7.03	0.879
	Difference	0.8±1.23	4.83±4.11	0.005
180 days	MMSE score	20.6±5.62	23.08±6.47	0.877
	Difference	0.9±1.66	4.92±4.81	0.029

Difference = MMSE score (Days after treatment - Day 0); *comparison between control and transplantation group.

porting therapeutic advantages of hUC-MSCs treatment in functional recovery after HIE.

Assessment of cognitive function and emotional reactivity

The mini-mental state examination (MMSE) was performed to measure the outcome of cognitive impairment in HIE. Similar MMSE scores were found between the two groups at day 0 (P = 0.895). Improvement of cognition ability was barely observed in the control group during the follow-up period. Conversely, prominent recovery was achieved in patients with stem cell treatment, which was indicated by the significance of therapeutic differences (P<0.05) at all designated time points between the two groups (**Table 4**).

The Hamilton Anxiety Rating Scale (HAMA) and the Hamilton Rating Scale for Depression (HRSD) were used to rate the severity of patients' anxiety (**Table 5**) and depression (**Table 6**), respectively. Although no significant difference was observed in long-term outcomes of emotional sufferings, we found remarkable improvement of both anxiety (P = 0.026) and depression (P = 0.022) in the early stage (day 14) of disease after cell therapy.

Assessment of extrapyramidal tract function

Unified Parkinson's Disease Rating Scale (UPDRS) was performed to evaluate extrapyramidal tract function in HIE patients (**Table 7**). No difference in UPDRS was observed between the two groups before the application of cell therapy (P = 0.805). Compared with control HIE patients, hUC-MSCs therapy significantly improved UPDRS ratings at day 90 (P = 0.033) and day 180 (P = 0.017) post transplantation.

Adverse effects of hUC-MSCs transplantation

The intravenous infusion of allogeneic hUC-MSCs in patients with HIE was feasible, safe, and well-tolerated. The incidence of adverse events was similar across the two groups. There were no significant adverse effects observed in all patients with hUC-MSCs transplantation, such as fever, variation in blood pressure, allergic reactions and so on. The vital signs of all enrolled subjects were under normal limits. No significant difference of other laboratory indexes were observed bet-

Table 5. $HAMA_{24}$ score of patients with HIE pre- and post-transplantation (mean ± sd.)

Time	Variables	Control group n = 10	Transplantation group n = 12	*P**
0 day	$HAMA_{24}$ score	11.7±5.7	10.36±4.46	0.671
14 days	$HAMA_{24}$ score	8.7±4.74	5.55±3.64	0.276
	Difference	-3±1.33	-4.82±2.04	0.026
90 days	$HAMA_{24}$ score	8.5±4.93	6.45±4.3	0.400
	Difference	-3.2±1.4	-3.91±4.83	0.650
180 days	$HAMA_{24}$ score	8.5±5.13	6.27±4.52	0.383
	Difference	-3.2±1.32	-4.09±5.15	0.590

Difference = $HAMA_{24}$ score (Days after treatment - Day 0); *comparison between control and transplantation group.

Table 6. $HAMD_{14}$ score of patients with HIE pre- and post-transplantation (mean ± sd.)

Time	Variables	Control group n = 10	Transplantation group n = 12	*P**
0 day	$HAMD_{14}$ score	10.5±5.8	9.36±4.78	0.628
14 days	$HAMD_{14}$ score	8.2±4.66	4.82±3.09	0.269
	Difference	-2.3±2	-4.55±2.11	0.022
90 days	$HAMD_{14}$ score	7.8±4.39	5.64±4.67	0.435
	Difference	-2.7±2.31	-3.73±3.13	0.407
180 days	$HAMD_{14}$ score	7.6±4.67	4.91±4.78	0.368
	Difference	-2.9±1.6	-4.45±4.18	0.273

Difference = $HAMD_{14}$ score (Days after treatment - Day 0); *comparison between control and transplantation group.

Table 7. UPDRS score of patients with HIE pre- and post-transplantation (mean ± sd.)

Time	Variables	Control group n = 10	Transplantation group n = 12	*P**
0 day	UPDRS score	28.9±12.67	27.5±13.39	0.805
14 days	UPDRS score	24.1±11.41	19.75±10.49	0.579
	Difference	-4.8±2.35	-7.75±4.63	0.058
90 days	UPDRS score	23.8±10.61	17.08±11.85	0.438
	Difference	-5.1±4.33	-10.4±6.19	0.033
180 days	UPDRS score	23.5±10.41	16.42±12.62	0.423
	Difference	-5.4±3.37	-11.1±6.47	0.017

Difference = UPDRS score (Days after treatment - Day 0); *comparison between control and transplantation group.

ween the two groups, including routine blood test, glycemia, liver function and renal function.

Discussion

hUC-MSCs are multipotent stem cells that can differentiate to various cell types under suitable conditions [5]. Human umbilical cord is an ideal source of stem cells because of its accessibility, abundant resources and painless procedures for harvesting [8]. The safety of transplanted MSCs is well documented in animal models but remains barely reported in humans [16]. Previous studies in rodent models [17] and nonhuman primates have showed no transplantation-related toxicity after intravenous infusion of hUC-MSCs [18]. In line with recent clinical findings in ischemic myocardiopathy [19], we showed that hUC-MSC transplantation through the peripheral vein is feasible and safe with little side effects. Further clinical trials with extended follow-up observation should be conducted to assure the long-term safety of hUC-MSCs transplantation.

MSCs are known as multipotent adult progenitor cells with a broad potential of differentiating into multiple cell types. Accumulative findings have demonstrated that induced neuron-like cells from MSCs undergo neuronal morphologic changes and express diverse neuron-specific markers, including nestin, β-tubulin III, neurofilament, vimentin and glial fibrillary acidic protein [20, 21]. Recent studies confirm similar differentiation potential in hUC-MSCs [22]. When exposed to neuronal conditioned medium, hUC-MSCs can differentiate into microglial cells, generate neuronal proteins, and upregulate the astrocyte protein glial fibrillary acidic protein [23, 24]. Long-term neuronal differentiation of hUC-MSCs can be induced as well by an exposure to neuronal induction chemicals like potassium chloride, valproic acid, forskolin, hydrocortisone, and insulin [25]. Transplanted hUC-MSCs might differentiate into neurons, astrocytes, and glial cells in a rat model of stroke and promote neuroplasticity [26]. The neural differentiation potential of hUC-MSCs holds tremendous interest for their application in neurological disorders. For example, emerging data suggest that hUC-MSCs are a good source of dopaminergic neuron-like cells, which represent a new strategy of stem cell therapy for neurodegenerative disor-

ders such as Parkinson's disease (PD) [27, 28]. After transplantation into different animal models of PD, these neuron-like cells could perform the physiological functions of dopaminergic neurons and play a therapeutic role to ameliorate the symptoms of PD [22, 27]. One intriguing question is whether the transplanted hUC-MSCs could migrate to involved nervous system and replace the injured neurons via differentiation. Although transplanted hUC-MSCs could be tracked in the striatum 4 months after microinjection into the PD rats [29], direct evidence remains lacking at present that transplanted cells could exert normal neuronal functions.

Besides the potential of neuron differentiation, solid evidence suggests that hUC-MSCs can directly protect neural tissues through paracrine mechanisms [30], which play a prominent role in the recovery of neurological disorder. Early transplantation experiment indicates that neurologic benefit resulting from intravenous infusion of hMSCs may derive from the increase of brain-derived neurotrophic factor and nerve growth factor in the ischemic tissue [31]. Recent study further demonstrates that treatment with hUC-MSCs can facilitate functional recovery after traumatic spinal cord injury [32]. In this study, implanted hUC-MSCs survived, migrated over short distances in the infarct cortex and released vast neuroprotective and growth-associated cytokines, including glial cell line-derived neurotrophic factor and neurotrophin-3 in the host spinal cord. Notably, implantation of human MSCs, even though a limited number of the cells engrafted, can stimulate proliferation, migration, and differentiation of the host endogenous neural stem cells via paracrine of beneficial growth factors [33].

Furthermore, hUC-MSCs are generally considered as immunoprivileged cells because they are MHC class I (MHCI) dull and negative for MHC class II (MHCII). Therefore, hUC-MSCs can serve as important immune regulatory cells that mediate protective immunity in neurological disorders after transplantation. Recent findings demonstrate that transplanted hUC-MSCs can suppress T cells, B-cells and natural killer cells and steer monocytes and dendritic cells to an immature state [5]. Similar findings from animal studies have confirmed the immunoregulatory capacity of transplanted MSCs in diverse autoimmune neurological disorders [34]. However, this therapeutic effect have been reported exclusively when MSCs were administered at disease onset or at the peak of disease, but unlikely improved neurological deficits when the disease was already in the chronic progressive phase [34]. One possible explanation is hUC-MSCs can be activated to increase MHCI and to express MHCII with IFN-gamma stimulation. When injected in an inflamed region, injected repeatedly in the same region or stimulated with IFN-gamma prior to injection, hUC-MSCs could be turned into immunogenic and thus loss their immunosuppressive capacity [35]. In this study, we performed cell infusion once per week for the first three-week. Although the immunogenicity of injected cells could not be directly assessed, no difference was observed in routine blood examination and allergic reactions after transplantation. Further study remains necessary to evaluate the long-term effects of hUC-MSCs transplantation, particularly with repeated infusion, on the host neuroimmune system.

Recent findings in animal studies have supported a potential therapeutic role of implanted hUC-MSCs in ischemic stroke [36, 37]. Rat models of stroke with hUC-MSCs implantation exhibit a trend toward less infarct volume and less atrophy than the control group, which is mostly attributed to vast production of neural growth-promoting factors from engrafted cells [38]. These preclinical studies strongly support that the therapeutic effects afforded by hUC-MSCs transplantation are related to dynamic paracrine and immune interactions between transplanted cells and host cells. Importantly, hUC-MSCs treatment is able to improve long-term neuronal recovery after ischemic stroke. Delayed administration of hUC-MSCs, even up to 30 days post-stroke, can improve neurological functional recovery in a rodent model of focal ischemia [39]. In this study, we evaluated the safety and therapeutic efficacy of hUC-MSCs therapy in patients with HIE. Clinical ratings of neurological function, extrapyramidal tract function, cognitive function and emotional reactivity were performed after cells infusion through a 180-day follow-up. Better neurological recovery has been achieved in HIE patients with hUC-MSCs transplantation than control subjects, particularly at day 180 post-transplantation. No significant side effects or com-

plications of cell transplantation were found in all subjects with hUC-MSCs treatment. Our data thus suggest hUC-MSCs hold promising potentials of novel therapeutic strategies for HIE and other ischemic neurodegenerative disease.

At the same time, we do recognize that our study has several limitations. First, as an open-label study design, our results may be prone to observer bias, which could be minimized by use of clearly defined assessment tools. Second, this is a single-center study performed in a limited number of subjects of Han Chinese ethnicity, which might be insufficient to draw the same conclusion in other populations. In addition, future study with extended follow-up remains necessary to evaluate long-term safety and efficacy of hUC-MSCs therapy. Thirdly, we did not track the hUC-MSCs in patients after transplantation, which however remains technically challenging in clinical trials. Lastly, it is worth noting that applied protocols for stem cells culture and transplantation are fundamental for therapeutic outcomes. There is currently no consensus regarding the cell culture condition, optimal infusion dose, mode of delivery and the timing of hUC-MSCs treatment. This study was not designed to compare the possible influence of different techniques and procedures on the clinical outcomes of stem cell therapy. However, the technique and procedure performed in this study have been widely used in many other published reports.

Conclusion

In conclusion, to our knowledge, this is the first clinical study specifically designed to investigate the outcomes of hUC-MSCs therapy in patients with HIE. Our data suggest that intravenous infusion of hUC-MSCs is safe and effective for the neurological recovery of HIE patients. Large well-designed RCTs with multicenter collaboration and long-term evaluation should be recommended to provide more independent information before hUC-MSCs implantation can be used as a routinely applied clinical therapy for ischemic neurodegenerative disease in general.

Methods and materials

Patients

This study was open-labeled, single group assigned and controlled. It was registered at ClinicalTrial.gov of the National Institute of Health of the USA (registered No. NCT01962233). And it was approved by the Ethics Committee of the First Hospital of Hebei Medical University. A total of 22 ischemic encephalopathy patients were assigned into either the treatment group or control groups. Inclusion Criteria: Patients are screened for enrollment in the study if both clinical signs and laboratory tests meet the diagnosis standards recommended by ICD-10 about hypoxic ischemic encephalopathy. Exclusion Criteria: Exclusion Criteria include diagnosis of major diseases in liver, kidney, and heart. Additional exclusion criteria include pregnancy, use of immunosuppressive medication, tumor, viral diseases and other immunodeficiency diseases. The same conventional treatment was performed to the two groups of HIE patients, including lowering the intracranial pressure, trophic nerve, hyperbaric oxygen, acupuncture, and rehabilitation. On the basis of conventional therapy, HIE patients in the transplantation group received a single intravenous infusion of hUC-MSCs while the control subjects received saline infusion. Intensive monitoring was applied to all subjects in the first 72-hour after transfusion, including vital signs and other adverse reactions like allergy.

Preparation and identification of hUC-MSCs

Informed consent for umbilical cord was obtained from the donors with research purposes. hUC-MSCs were prepared according to the previous study [40]. Briefly, the arteries, veins, and epithelium of umbilical cord were removed. The mesenchymal tissue was diced into cubes and washed. Theobtained tissue was minced into fragments of approximately 0.5 cm^3 and centrifuged at 250 g for 5 minutes. Following removal of the supernatant, these fragments were washed with 0.1 M PBS and centrifuged at 250 g for 5 minutes. Finally, the fragments were seeded into a T75-cm^2 tissue culture flask. After 10-12 days of culture, the remnants of the cord fragments were removed, and the adherent cells were cultured to 80% confluence (first passage). hUC-MSCs were then expanded and collected between the third and fifth passage for transfusion into patients. Before transfusion, the hUC-MSCs were subjected to quality control. The hUC-MSCs were digested and labelled with anti-CD34-PE, anti-CD44-PerCP-Cy5.5, anti-CD45-PerCP-Cy5.5, anti-CD73-APC and anti-HLA-DR

FITC. The cells were next sorted using FACSCalibur flow cytometer and the data were analyzed by FlowJo software. hUC-MSCs obtained between passages 6 and 8 were used for transplantation.

hUC-MSCs transfusion

Prior to transplantation applications, bacteriological and endotoxin tests were performed on the hUC-MSCs. Cell viability was detected by Trypan blue. hUC-MSCs containing 1×10^8 cells were suspended in 100 ml saline and intravenously infused to patients. The transfusion time was no more than 30 minutes. The control patients received the same volume of saline. Transfusion of hUC-MSCs or saline was administered to subjects once a week for three weeks. The data of all enrolled subjects were obtained through a 180-day follow-up period of observation.

Clinical assessment

The clinical assessments were performed before and after treatment for 14 days, 90 days and 180 days, respectively. The National Institutes of Health Stroke Scale (NIHSS) and Barthel Index were used to evaluate neurological function of patients. The motion part of Unified Parkinson's Disease Rating Scale (UPDRS) was used in the evaluation of extrapyramidal tract function. The cognitive function and emotional reactivity were assessed by mini-mental state examination (MMSE), Hamilton Depression Scale 14 Item ($HAMD_{14}$) and Hamilton Anxiety Scale 24 Item ($HAMA_{24}$). Clinical data were collected at each designated time point, including basic vital signs, routine blood test, glycemia, liver function examination, renal function examination and so on

Statistical analysis

Continuous variables that was on per-treatment and change of post-treatment were compared by the Student t-test between control and treatment groups, repeated ANOVA were performed for comparison on continuous variables of post-treatment between control and treatment groups. Categorical variable of two groups were compared by Pearson's χ^2-test. Values of $P<0.05$ were considered as statistically significant. All statistical analyses were performed using SAS 9.3.

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Disclosure of conflict of interest

None.

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