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Author(s): Zhong-Min Li, Zhi-Ti Zhang, Chuan-Jun Guo, Feng-Yang Geng, Fu Qiang, Le-Xin Wang

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Autologous bone marrow mononuclear cell implantation for intracerebral hemorrhage- a prospective clinical observation

Zhong-Min Li,^a Zhi-Ti Zhang,^a Chuan-Jun Guo,^a Feng-Yang Geng,^a Fu Qiang,^a Le-Xin Wang^b

a. Department of Neurosurgery, Liaocheng People's Hospital of Taishan Medical University, Liaocheng, Shandong, 252000, P.R. China

b. School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW 2678, Australia.

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Author for correspondence:

Prof Lexin Wang, School of Biomedical Sciences, Charles Sturt University
Wagga Wagga, NSW 2678, Australia.

Phone: +61 2 69332905, Fax: +61 2 69 332587, E-mail: lwang@csu.edu.au

OR

Dr Zhong-Min Li, Department of Neurosurgery, Liaocheng People's Hospital, Liaocheng, Shandong, 252000, P.R. China. Email: lizhm1@163.com

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Abstract

Background: This study was designed to assess the clinical effect of bone marrow mononuclear cells including mesenchymal stem cell (MSCs) in patients with intracerebral hemorrhage (ICH). **Methods:** One-hundred patients were divided into a study (n=60) or a control group (n=40). Bone marrow mononuclear cells from the same patient were injected to the perihemorrhage area in the base ganglia through an intracranial drainage tube 5.9 days after ICH. National Institute Stroke Scale (NIHSS) and Barthel index was used to assess neurologic impairment and daily activities, respectively, before and 6 months after intervention. **Results:** Six months after implantation, the NIHSS score in the study group was lower than in the control group (10.09 ± 8.86 vs 14.35 ± 10.14 , $P < 0.01$), whereas the Barthel scores were higher (57.39 ± 23.51 vs 46.90 ± 20.29 , $P < 0.01$). Neurological and functional improvement was observed in 52 (86.7%) of the study group patients, and in 17 (42.5%) of the control group patients ($P = 0.001$). No allergic or other adverse effects were observed in the study group. **Conclusion:** Autologous bone marrow mononuclear cell implantation reduced neurological impairment and improved activities of daily living in a selected group of ICH patients. Further studies are required to ascertain the long-term safety and efficacy of this treatment.

Keywords: bone marrow; mesenchymal stem cell; intracerebral hemorrhage; stroke.

Introduction

Intracerebral hemorrhage (ICH) is a life-threatening form of stroke and represents more than 15% of all strokes.^{1,2} ICH is associated with a high mortality and high incidence of long-term neurological disability in survivors.^{1,2} Current treatment strategies for ICH include surgical evacuation of hematoma, reduction of intracranial pressure, prevention of cerebral edema and general supportive measures.³ The effectiveness of these therapeutic measures is limited, and new and more effective approaches are required.³

Human bone marrow contains hematopoietic stem cells and mesenchymal stem cells (MSCs). MSCs possess self-renewal capacity and pluripotency defined by their ability to differentiate into bone, fat, cartilage and muscle.^{4,5} MSCs are also known to differentiate into neurons and glial cells *in vitro* and *in vivo*.^{6,7} A recently introduced permanent and stable human MSC lines, B10 human MSCs, has been found to differentiate into neural cell types including neural stem cells, neurons, astrocytes and oligodendrocytes *in vitro*.⁸

Following brain transplantation in ICH mouse, B10 human MSCs integrate into host brain, survive, differentiate into neurons and astrocytes and induce behavioral improvement.⁸

Several other studies on ICH animals also showed that when MSCs are delivered to the brain through carotid artery or direct intracerebral injection, they differentiate into neurons and astrocytes in the areas surrounding the bleeding foci, and improve motor activity and neurological function recovery.^{9,10} So far there has been little information about the actions of bone marrow mononuclear cells or MSCs in human brains after hemorrhagic stroke. The purpose of this study was to investigate the clinical effect of autologous bone marrow mononuclear cell implantation in patients with ICH.

Patients and methods

Patient selection

This study was approved by the institution review board of Liaocheng People's Hospital and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Informed consent was obtained from all participants or their relatives. For patients with speech or language difficulties, the initial consent at the screening stage, and the full consent before cell implantation was given by the patient's immediately family. Between January 2008 and January 2010, 500 patients who were admitted to the Department of Neurosurgery due to acute ICH were prospectively screened. Four hundred were excluded for not meeting the selection criteria (n=325) or refused to participate in this study (n=75). The diagnosis of acute ICH was based on symptoms of focal neurological deficit not related to traumatic head injury, and was confirmed by MRI and CT scan. The selection criteria included all of the following: 1) hypertensive ICH with onset of symptoms ≤ 6 h; 2) <80 years old; 3) Glasgow Coma Scale score of 5-12; 4) ICH location on CT and MRI was limited to above tentorium and without significant intraventricular extension; and 5) indications for decompression surgery: ICH volume > 20 mL on CT scan and >10 mm shift of brain from the midline. Exclusion criteria included any one of the following: 1) ICH caused by factors other than hypertension, such as head injuries, anticoagulants or tumor; 2) a history of allergy; 3) mild ICH focal neurological deficits with no indication for decompressive craniotomy or surgical evacuation of hematoma; 4) concurrent chronic illnesses such as hepatic or renal dysfunction; 5) coagulation disorders; 6) unable to give a written informed consent at the screening stage.

Five days after surgical drainage and decompressive craniotomy, the selected patients and their relatives were further consulted on the potential benefits and risks of this mononuclear cell treatment, with particular emphasis on the uncertainties in its clinical effects and long-term side effects. In the end, full written consent on the cell implantation was obtained from 63 patients. The other 42 patients who did not wish to receive bone marrow mononuclear cell treatment participated in this study as the control group. The baseline characteristics of the patients are shown in Table 1.

Surgical treatment

All patients underwent careful neurologic evaluations and deemed as necessary for decompression surgery by using the standard protocol of our department. The methods for surgical decompression were determined by the location and volume of the intracerebral hemorrhage as well as intracranial pressure. This could be hematoma evacuation through craniotomy or decompression through a small window on the skull. In all patients, a tube was placed in the hematoma cavity for drainage after the surgery.

Bone marrow mononuclear cell preparation

Bone marrow extraction was performed on the day of mononuclear cell treatment, approximately 5 days after ICH. Under local anesthesia, 200 ml bone marrow was aspirated from the posterior iliac crest of patients in the study group. The bone marrow was placed in a testing tube containing sodium citrate. Mononuclear cell cells were extracted by mononuclear cell isolating reagent (Zhonglianda Biotechnology, Ningxia, China), using the methods

specified by the manufacturers. The mechanism of mononuclear cell extraction is to use hydroxyethyl starch for erythrocyte sedimentation, and Ficoll–Hypaque density gradient centrifugation to isolate mononuclear cells.¹¹ About 0.5 ml mononuclear cell suspension was obtained after centrifuging and washing with normal saline. Gentamicin was added to the mononuclear cell suspension which was diluted to 4.0 ml with normal saline. A small volume (0.5 ml) of the diluted cell suspension was sent to the central laboratory for cell counting and viability testing by trypan blue exclusion. The cell suspension (5-10 μ L) was placed in a cell counting device, and the number of cells in the 4 large squares was counted by an automatic blood cell counter (SEAC, Genius, Italy). The cell number was calculated using the following formula: Cells/L= (cells in 4 large squares/4) x 10 x 8 x 10⁶. MSC count was performed by flow cytometry. After labeling with relevant antibodies, CD44 +, CD105 +, CD14-, CD34- cells were counted, and total number of MSCs was calculated. The remaining 3.5mL diluted cell suspension was injected into the brain, 5-7 days after the onset of ICH, via a drainage tube positioned during the initial decompressive surgery. The tube was re-positioned to the peripheral area of the hematoma cavity before the injection. For the control group patients, approximately 3.5 mL of normal saline was injected through the drainage tube 5-7 days after the onset of ICH.

Post-implantation care

Patients were closely monitored in the intensive care unit for 5-8 h and then in the ward for further 3-5 days. Antibiotics were routinely used for 3 days after the mononuclear cell implantation. Control group patients also received the same prophylactic antibiotics for 3-5

days as they were also treated surgically for simple drainage or hematoma evacuation.

Patients were discharged to their own home but were followed up in our clinics. All patients participated the standard rehabilitation programs in our clinics, including physiotherapy, exercises, and language training. Antihypertensive medications (mostly calcium channel blockers and angiotensin-converting enzyme inhibitors) were prescribed to all patients.

Assessment of neurological function

Five days after surgical drainage of ICH, the NIH Stroke Scale (NIHSS) assessment was performed in the study group (24 h before the mononuclear cell implantation) and control group patients to quantitatively assess neurologic deficit. NIHSS was performed again 6 months after the first assessment. NIHSS is an 11-item neurologic examination scale used to evaluate the levels of consciousness, language, visual-field loss, extraocular movement, motor strength, ataxia, dysarthria, and sensory loss. A trained neurologist who was unaware of the study groupings rated the patient's ability to answer questions and perform activities. Ratings for each item were scored with 0 to 2 or 3 grades with 0 as normal. The Barthel index, a 10-item instrument, was also used five days after the surgical ICH treatment, and 6 months after the first assessment to evaluate the patient's activities of daily living and mobility. The items included feeding, moving between wheelchair and bed, grooming, bathing, walking on level surface, going up and down stairs, dressing, continence of bowels and bladder. A higher score denotes a more independent living.¹²

Six months after the mononuclear cell implantation, the patient's swallowing difficulty, muscle strength, muscle tension, language ability, calculation, cognition, and responsiveness

to painful stimulation were separately evaluated by a neurologist who was blinded to patient's treatment. Affected limb muscle strength was classified into 5 grades, with grade 0 being complete paralysis, grade IV being weakened muscle strength but can move against resistance. Muscle tension of the affected limbs was assessed by manual palpitation and was compared with that of the unaffected limbs. Cognition was assessed by Mini-Mental State Examination.¹³ Language ability was assessed by the use of the American Speech-Language-Hearing Association Functional Assessment of Communication Skills for Adults.¹⁴ Calculation ability was assessed by a set of mathematical questions of addition, subtraction, multiplication and division. Responsiveness to painful stimulation was evaluated by pressing an examination pin to the upper and lower extremities of the affected and unaffected sides of the body. The neurologists who performed the neurological function assessments were unaware of patient's groupings or clinical treatment throughout this study.

Statistical analysis

Data were expressed as means \pm SD. SPSS13.0 software was used for data analysis. Numerical data were analyzed with one-way ANOVA. Categorical data were analysed by Fisher's exact test. Two tailed $P < 0.05$ was considered statistically significant.

Results

General findings

All patients in the study and the control group survived. Three patients from the study group and two from the control did not return to the hospital for schedule follow-up, and were

excluded from the final analysis. In the end, 60 patients in the study group and 40 in the control group were included in the final analysis.

Intracranial hemorrhage was located in the brain basal ganglia in all patients. There was no significant difference in the baseline characteristics including neurological findings and the mean volumes of bleeding between the two groups ($P > 0.05$, Table 1). Simple drainage, evacuation of hematoma or decompressive craniotomy was performed in all patients, with no significant difference in the surgical methods between the two groups ($P > 0.05$, Table 2). The time from the onset of ICH to surgery was also similar between the two group ($P > 0.05$, Table 2).

At the end of follow up, there were no signs of re-bleeding or infection in both groups. There was no significant difference in the mean systolic (142.3 ± 9.2 vs 140.6 ± 7.5 mm Hg, $P > 0.05$) or mean diastolic blood pressure (83.6 ± 6.9 vs 85.1 ± 9.9 mm Hg, $P > 0.05$) between the study and the control group. Using blood pressure $< 140/80$ mm Hg as a therapeutic target, the proportion of patients achieved blood pressure control in the study and the control group was 72% (43/60) and 68 (27/40), respectively ($P > 0.05$)

All cells in the diluted cell suspension were mononuclear cells. The median nucleated cell count in the diluted cell suspension of the 60 patients, which included the MSCs, was $3.79 \times 10^9/L$ (range, 7.00×10^8 to $6.53 \times 10^9/L$). Cell viability was found to be over 95% in each sample. The median number of MSCs injected to each patient was $9.47 \times 10^5/L$ (range, 7.25×10^5 to $1.35 \times 10^6 /L$). The mean time from the onset of ICH to stem cell implantation was 5.9 ± 0.6 days.

Neurological assessment

There was no significant difference in the baseline NIHSS and Barthel scores between the two groups (Table 3). After MSC implantation, NIHSS scores in the study group were reduced, and Barthel scores were increased ($P < 0.01$). There was also a reduction in NIHSS and an increase in Barthel scores in the control group, but the mean NIHSS scores was higher, whereas the mean Barthel scores were lower than in the study group ($P < 0.01$).

The proportion of patients who experienced complete recovery from pre-treatment swallowing difficulties, reduced muscle strength or tension, compromised language and cognition, or reduced responses to painful stimulation are listed in Table 4. A higher rate of complete recovery in the above indices was found in the study (Table 4). Improvement in one or more of the above neurological and functional assessment measures was observed in 52 (86.7%) study group patients, and in 17 control group patients (42.5%, $P = 0.001$).

Side effects

Low grade fever (38.5°C) was noted in 5 (5.3%) patients from the study group within the first 24 h of implantation. One patient (2.5%) in the control group also experienced low grade fever. The fever in all 6 patients subsided in 3 days without specific pharmacological intervention. One patient in the study group presented to the clinic with continuous dull chest pain 4 months after the stem cell implantation. Chest CT showed lung cancer in this patient.

Discussion

Earlier *in vivo* animal studies on bone marrow stem cells and brain injuries were mainly centered on ischemic stroke. Bone marrow stem cells, when delivered by intravenous or intracerebral injection, migrate to the ischemic sites and differentiate into neuron cells. Bone marrow stem cell injections in these animal models ameliorated neurological defects and improved neurological functionality.¹⁵⁻¹⁷ Recent evidence showed that in rats with hemorrhagic stroke, bone marrow MSCs also improves motor activities and function few weeks after the treatment.^{9,10} In a rat ICH model, intracerebral injection of allogeneic bone marrow stromal cells donor cells enhances endogenous neurogenesis and inhibits apoptosis of newborn neural cells.¹⁸ In a monkey ICH model, intracerebral implantation of human mesenchymal stem cells was associated with a higher vessel density in the area of hematoma and improved recovery of neurological function.¹⁹

Clinical studies on bone marrow stem cells and stroke are scarce, and the effect of bone marrow MSCs on ICH patients is unknown. In a small randomized trial, autologous MSCs were infused intravenously to 5 patients with ischemic stroke.²⁰ Consistent improvement in neurological function was detected in the 5 patients one year after the onset of stroke.¹⁶ Series evaluations showed no adverse effects from MSCs treatment.²⁰ In a more recent study, bone marrow stem cells were injected directly into the perilesional area in 5 stroke patients.²¹ After one year follow up, there was some improvement in neurological function but no serious adverse effects.²¹

In the present study, bone marrow mononuclear cells including MSCs were injected directly to the perilesional areas of the hemorrhage. Six month follow up in these patients showed greater improvement in NIHSS and Barthel index scores than in the control group

patients. A higher proportion of study group patients showed complete recovery in other neurological indices, such as swallowing and language skills, muscle strengths and tension, calculation and intelligence, as well as responses to stimulation. Improvement in one or more of the above neurological and functional assessment measures, swallowing difficulty, muscle strength, muscle tension, language and cognition, and responses to painful stimulation, was observed in 86.7% of the study group and 42.5% of the control group. These results clearly demonstrate that mononuclear cell implantation is associated with a better neurological recovery following hemorrhagic stroke.

The most important aspect in any cell implantation technique is that the chosen cells are able to proliferate *in vivo*, and structurally and functionally integrate into the brain. Neural stem cells, human umbilical cord blood cells, adult tissue specific stem cells and bone marrow stromal cells have been tried in various animal and human studies on stroke. Bone marrow stem cells or bone marrow MSCs appear to have several advantages over other cell types for clinical treatment of stroke. Autologous bone marrow mononuclear cells including MSCs can be easily obtained from the same patient, and implanted shortly after decompressive surgeries. These cells differentiate relatively quickly *in vitro*,²² and when given by intravenous infusion, can come cross blood brain barriers.²³ In addition, allergic reactions and other adverse effects in humans appeared to be rare with this cell type.^{20, 21, 24} In the present study, 5 mononuclear cell-treated patients experienced low-grade fever which subsided in 3 days without pharmacological intervention. The reasons for the fever were unclear but allergic reactions are unlikely, as autologous stem cell implantation is not known to cause allergies in human recipients in short or long term.^{20, 21, 24} One patient was found to

have lung cancer 4 months after the MSCs implantation. There is no direct evidence to suggest that bone marrow mononuclear cells may lead to lung or other cancer, but further follow up study is required to confirm the long-term safety of this treatment.

The appropriate time for stem cell implantation after ICH is unknown. In the acute setting, release of excitotoxic neurotransmitters, free radicals, and proinflammatory mediators might threaten new tissues introduced into the infarct or hemorrhagic region. On the other hand, the effect of delayed stem cell implantation several weeks after stroke may be adversely affected by the formation of scar tissues. In animal studies on hemorrhagic stroke, stem cell implantation was often conducted 12 h after the induction of ICH.^{9,10} In the present study, we performed mononuclear cell implantation after drainage of the intracerebral blood, on average 5.9 days after the onset of ICH. This timing was programmed with the time for drainage tube removal, and was convenient to the patients and the operators. Although the current regimen is clinically effective, the optimal timing for the stem cell implantation is yet to be established.

One limitation of this study was that apart from MSCs, other hematopoietic cells such as progenitor cells were also injected to the perilesional area of the brain hemorrhage. The precise cell types responsible for the clinical effects observed in this study require further investigation. Also, this study was based on a highly selective group of patients with a low mortality rate and low re-bleeding rate. Whether this mononuclear cell therapy is effective in all ICH patients remains to be seen. Finally, due to the experimental nature of the stem cell treatment, we were unable to conduct a randomized clinical trial. We used patients who declined to receive stem cell therapy as a control group, which may cause bias in the final

analysis. Fortunately, there was no statistically significant difference in the baseline characteristics in the control and study group before the administration of MSCs (Table 1).

In conclusion, the present study demonstrated that intracerebral administration of bone marrow mononuclear cells including MSCs is safe in a selected group of patients with ICH. This mononuclear cell treatment is associated with a significant improvement in neurological function 6 months after the therapy. Further studies are required to ascertain the long-term safety and efficacy of this therapy.

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Table 1. Baseline characteristics.

	<i>Study group (n=60)</i>	<i>Control (n=40)</i>	<i>P</i>
Age (yrs)	56.3±2.9 (39-74)	55.9±4.7 (35-72)	0.422
Sex (M)	37 (61.7%)	23(57.5%)	0.683
Transient unconsciousness	33(55%)	29(72.5%)	0.077
Language impairment	35(58.3%)	20(50%)	0.558
Loss of mobility	57(95%)	35(87.5%)	0.328
Affected limb muscle strength			
Grade 0	25(41.7%)	19(47.5%)	0.565
Grade I-II	24(40%)	15(37.5%)	0.799
Grade III-IV	11(18.3%)	6(15%)	0.664
Bleeding volume on CT scan			
20-30 mL	20(33.3%)	18(45%)	0.239
	24.2 ± 2.2 mL	26.0 ± 3.2 mL	0.842
30-50 mL	28(46.7%)	13(32.5%)	0.158
	39.5 ± 5.2 mL	37.9 ± 6.2 mL	0.766
>50 mL	12(20%)	9(22.5%)	0.764
	66.7 ± 5.2 mL	65.9 ± 4.8 mL	0.542
Glasgow coma scale score	10	10	0.229
(median)			

Numbers are expressed as means ± SD except Glasgow coma scale scores.

Table 2. Comparison of surgical management.

	<i>Study</i> (<i>n=60</i>)	<i>Control</i> (<i>n=40</i>)	<i>P</i>
Time from hemorrhage onset to surgery (h)	5.9 ± 1.2	5.7 ± 1.3	0.239
Simply drainage	24 (40%)	11(27.5%)	0.199
Hematoma evacuation through small skull window	28 (46.7%)	23(57.5%)	0.288
Hematoma evacuation through craniotomy	8 (13.3%)	6(15%)	0.814

Numbers are expressed as means ± SD.

Table 3. NIHSS and Barthel scores before and 6 month after transplant.

	<i>Study (n=60)</i>	<i>Control (n=40)</i>	<i>P</i>
NIHSS			
Post-operative	19.77±11.31	18.89±11.20	0.7028
After therapy	10.09±8.86*	14.35±10.14*	0.0086
Barthel score			
Post-operative	27.91±13.11	29.14±15.10	0.6664
After therapy	57.39±23.51*	46.90±20.29*	0.0032

**P* <0.01 compared with the baseline values in the same group. Numbers are expressed as means ± SD.

Table 4. Complete post-transplant recovery of neurological functions in patients who had significant impairment after intracerebral hemorrhage.

	<i>Study</i>	<i>Control</i>	<i>P value</i>
Swallowing	7/36 (19.4%)	2/27(7.4%)	0.044
Muscle tension	13/52(25.0%)	1/32(3.1%)	0.014
Muscle strength	15/56(26.8%)	1/23(4.3%)	0.003
Language	9/35(25.7%)	1/20(5.0%)	0.007
Calculation	15/60(25.0%)	0/33(0%)	0.000
Cognition	10/48(20.8%)	1/27(3.8%)	0.045
Response to painful stimulation	5/21(23.8%)	1/28(3.7%)	0.007