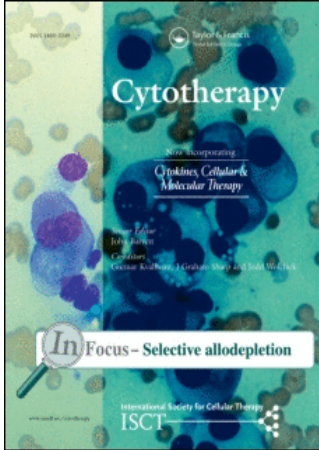


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# A combined procedure to deliver autologous mesenchymal stromal cells to patients with traumatic brain injury

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## Background

There is increasing evidence of therapeutic benefits from bone marrow (BM)-derived mesenchymal stromal cells (MSC) in various animal models with neurologic disorders. It is of great interest to apply the approach to clinical patients, i.e. to take the investigations from laboratory bench to the patient's bedside. This clinical trial was performed to assess the safety and feasibility of a combined procedure to deliver autologous MSC to patients with traumatic brain injury.

## Methods

MSC were isolated by BM aspiration and expanded in culture. Seven patients received autologous cell transplantation. A primary administration of  $10^7$ – $10^9$  cells was applied directly to the injured area during the cranial operation; a second dose of  $10^8$ – $10^{10}$  cells was infused intravenously. All patients were followed up regularly for 6 months.

## Results

There was no immediate or delayed toxicity related to the cell administration within the 6-month follow-up period. Neurologic function was significantly improved at 6 months after cell therapy.

## Discussion

The procedure used is safe and feasible at ordinary medical facilities without additional invasive procedures for the patient. The combined cell delivery procedure is expected to enhance the engraftment efficacy of transplanted cells at injured brain tissue, thereby promoting neurologic recover.

## Keywords

mesenchymal stromal cells, transplantation, traumatic brain injury.

## Introduction

Since bone marrow (BM)-derived mesenchymal stromal cells (MSC) were found to have differentiative plasticity, there has been great interest in their potential therapeutic application [1,2]. There is increasing evidence indicating a therapeutic benefit of MSC transplantation in various disorders characterized by cell injury or cell loss, such as myocardial infarction [3,4], stroke [5], Parkinson's disease [6] and liver disease [7]. The methods of MSC administration can be classified as directional, semi-directional and systemic delivery. Stereotaxic intrastriatal injection of MSC in a Parkinson's disease model [6] and local

administration of MSC in a freeze-induced brain damage model [8] are examples of directional delivery; intracarotid transplantation for stroke [9] and intracoronary injection for myocardial infarction [4] are considered semi-directional deliveries. In a stroke animal model, Chopp's group administered MSC intravenously [10], which is an example of systemic delivery. The safety and feasibility of autologous MSC administration have been verified by a variety of studies in animal models and a recent clinical investigation in stroke patients [11]. The therapeutic benefit of MSC is mainly determined by its homing number in the injured area, although the mechanism is

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complicated and very far from clear. The MSC homing efficiency in the injury site is closely related to the interval between injury and cell application and frequency of cell administration and relatively less related to the cell number of transplantation [12].

In the present study, we introduced a combined procedure to deliver autologous MSC to patients with traumatic brain injury (TBI). The feasibility and safety of this procedure were assessed for seven TBI patients. Neurologic function was also evaluated.

## Methods

### Patient selection

Six male patients, aged between 30 and 55 years, and one female patient, aged 6 years, were enrolled in this program. The procedure utilized in this clinical trial was approved by the Institutional Board of Medical Ethics at the Weifang People's hospital. Written informed consents were obtained from all involved patients or their parents. The inclusion criteria were as follows: traumatic brain injury with or without paralysis; a Barthel index (BI) value less than 40; a further operation required for the purpose of cranial correction or replacement; willingness to participate in the clinical trial.

### MSC isolation and *in vitro* expansion

MSC were isolated from the mononuclear cell population in the BM by a previously described method [13,14]. Briefly, under standard sterilization and local anesthesia, BM (10–15 mL) was aspirated from the posterior iliac crest using a heparinized syringe. Meanwhile, 30 mL venous blood was taken for serum extraction. The BM suspension was loaded on top of an equal volume of Ficoll (density 1.077 g/mL; Sigma, Shanghai, China) in a preloaded centrifuge tube and centrifuged at 1800 r.p.m. for 20 min. The top layer of mononuclear cells was collected and washed with Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen, Beijing, China) three times. The isolated cells were suspended in DMEM/15% fetal bovine serum (FBS) supplemented with hPDGF (10 ng/mL) and hEGF (10 ng/mL). The cells were seeded into 50-cm<sup>2</sup> flasks at a density of 10<sup>6</sup> cells/cm<sup>2</sup> and then cultured in a 95% humidified incubator at 37°C with 5% CO<sub>2</sub> in air. After 5 days, non-adherent cells were removed by replacing the medium. Attached cells developed into colonies within 3–7 days. When these primary cultures of MSC had reached 80–90% confluence, the cells were

harvested using 0.25% trypsin (Gibco) and subcultured at a ratio of 1:3 for three passages. After two passages of culture, FBS and growth factors were removed from the culture medium and replaced with 3% autologous serum. FBS was a local product from Hang Zhou Biology Technology Company (Hang Zhou, China), which was strictly screened and appraised by the China Medical Biotech Association (CMBA). It should be noted that not all regulatory authorities permit the use of FBS for clinical material. All other reagents used in this study were clinical grade.

### Viability assay, microbiologic tests and cytogenetic analysis

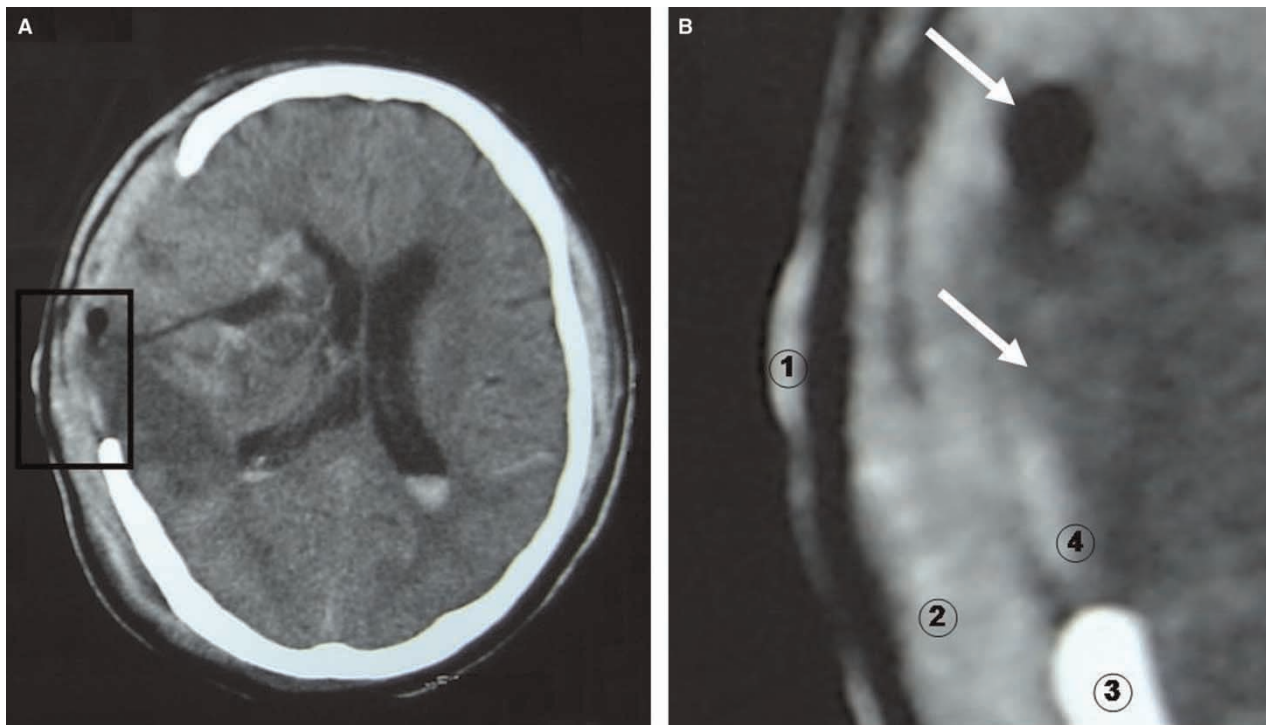
The cell viability was determined by trypan blue exclusion. Prior to transplantation, a sample of MSC suspension (10 µL containing 1 × 10<sup>4</sup> cells) was incubated with 5% trypan blue at room temperature for 5 min. The percentage of non-stained cells in the total number of cells was taken as the cell viability. A viability of ≥ 95% was one of the required criteria for the cells to be released for transplantation.

Microbiologic tests included Gram staining, testing the final medium culture for bacteria and fungi, and real-time polymerase chain reaction (PCR) detection for ureaplasma urealyticum (UU), chlamydia trachomatis (CT) and mycoplasma pneumonia (MP). Negative test reports of all microbiologic tests were required for MSC application.

After three passages of *in vitro* expansion, MSC retained normal cytogenetics, which was verified by conventional staining and G-banding techniques. The method of cytogenetic analysis has been described previously [14].

### Stem cell marker staining

To identify the stem cell population, harvested MSC were stained with saturating amounts of monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC): CD34 and CD45 (negative markers; Invitrogen, Burlington, Ontario, Canada) and CD44, CD90 and CD105 (positive markers; Invitrogen). The staining procedure was described in the manufacturer's instructions and applied to each individual subject. At least 20 000 events were analyzed by flow cytometry, using E6155 (BD FACS Calibur, Franklin Lakes, NJ, USA).



**Figure 1.** The location of direct administration of MSC in the brain. (A) The cross-section of a computed tomography (CT) scan. The box is enlarged in (B). The light arrows indicate injection sites, necrotic area and subarachnoid cavity. The numbers represent different layers of local structure: 1, skin; 2, temporalis muscle; 3, skull; 4, dura mater encephali.

### Procedures of MSC administration in TBI patients

Autologous MSC were administered with a combined procedure of directional and systemic deliveries, i.e. local direct application followed by intravenous infusion. Heparinization and filtration (40- $\mu\text{m}$  cell strainer; BD Falcon, Two Oak Park, Bedford, MA, USA) were carried out to prevent cell clotting and micro-embolization during intravenous transplantation. As shown in Figure 1, the MSC suspension was applied directly to the damaged area during the operation of cranial correction or replacement. After an interval of 4–12 days, the autologous transplantation was boosted by intravenous injection. Table 1 shows the MSC numbers of the first and second administrations for each patient.

### Neurologic severity and safety evaluations

The BI (Barthel Index) was used to evaluate the neurologic severity. BI is used as a simple index of independence to score the ability of a patient with a neuromuscular or musculoskeletal disorder to care for him- or herself and, by repeating the test periodically, to assess the patient's improvement. The values were determined upon a

patient's admission and during a 6-month follow-up period.

Although no serious side-effects or life-threatening events were expected from this autologous cell transplantation, a safety evaluation was undertaken based on IND regulations 21CFR 312.32. As described in the clinical

**Table 1.** Administered MSC dose for each patient

Patient	Age (years)	First dose (number of cells)	Second dose (number of cells)	Interval (days)
1	52	$1.66 \times 10^9$	$1.48 \times 10^8$	12
2	53	$1.16 \times 10^9$	$6.08 \times 10^8$	5
3	30	$6.63 \times 10^7$	$3.28 \times 10^9$	7
4	6	$8.74 \times 10^8$	$8.80 \times 10^{10}$	4
5	55	$9.35 \times 10^7$	$2.38 \times 10^8$	6
6	31	$1.62 \times 10^8$	$5.16 \times 10^8$	12
7	49	$1.16 \times 10^9$	$1.82 \times 10^{10}$	5

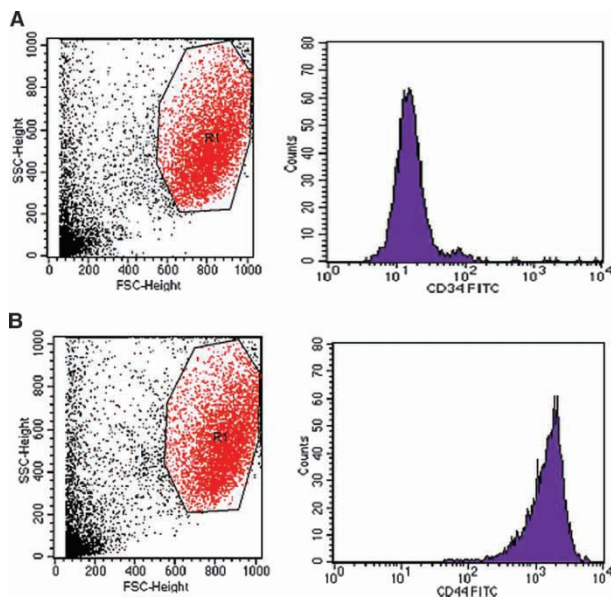
*The first administration was carried out locally by direct injection in a volume of 5–10 mL and the second dose was given by intravenous injection in a volume of 20 mL; the interval between the first and second administrations is indicated.*

study of Moviglia *et al.* [15], a complete clinical examination was performed before cell administration for each patient, and records of the patients' clinical, neurologic, cardiorespiratory, digestive and renal activity were documented. Physicians from the Department of Neurosurgery were designated to treat any adverse events (AE), with the power to discontinue the trial if needed. The evaluation of AE was based on the Common Terminology Criteria for Adverse Events v3.0 (CTCAE).

## Results

### Characterization of MSC

In human BM, the stem cell population is mainly composed of hematopoietic stem cells (HSC) and a very small non-HSC portion consists of MSC. Through the process of separation and *in vitro* expansion, a transplantable amount of MSC with relative purity was obtained within 2 weeks. Positive staining was observed on CD44, CD90 and CD109 markers, and CD34 and CD45 showed negative. The cell-marker distribution indicated that the preparation of MSC comprised the major part of the cell population prior to transplantation. Figure 2 shows a representative CD34 and CD44 detection.



**Figure 2.** Identification of a stem cell marker on MSC by flow cytometry. In the right panels, solid histograms represent the fluorescence intensity of human MSC with antibodies CD34 (A) and CD44 (B). The left panels show the cell populations (R1) from which the fluorescence intensity was counted.

MSC cytogenetic analysis showed a normal diploid karyotype (46XY or 46XX) and normal chromosome structure, which suggested that genetic stability was retained prior to autotransplantation.

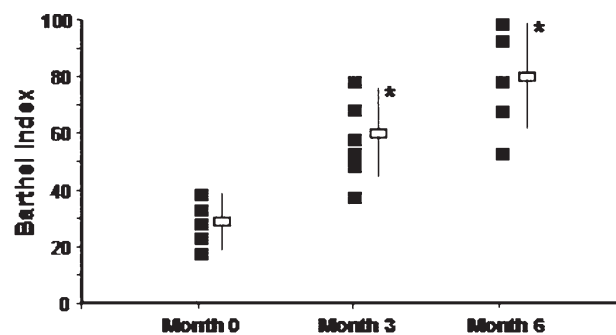
### Evaluation of the feasibility and safety of MSC administration

This combined procedure consisted of two steps, primary local direct application and secondary intravenous infusion. The first step was carried out during the cranial operation without adding extra invasive procedures. The second step was taken as a regular intravenous infusion. So the whole procedure was feasible for ordinary medical facilities.

Clinical and laboratory evaluations of the MSC-treated patients showed no deaths or cell-related serious AE. There was no immediate or delayed toxicity related to MSC administration within the 6-month follow-up period. One patient (patient 2) experienced epilepsy twice during the first 2 months. This patient was treated with phenytoin sodium and topamax and the symptoms did not occur again during the follow-up period.

### Assessment of functional recovery

The BI was assessed for each patient as a functional evaluation. As shown in Figure 3, neurologic severity was significantly improved by the third month and further improvement was observed at the sixth month following cell therapy.



**Figure 3.** Assessment of neurologic function of TBI patients. The solid square represents an assessed point for each patient at a different time. The open squares indicate the average ( $n = 7$ )  $\pm$  standard deviation and \* shows the significant difference ( $P \leq 0.05$ ) compared with month 0.

## Discussion

In the present study, MSC were administrated through a combined procedure of direct application followed by intravenous boost. This procedure proved to be both feasible and safe for all the patients involved with TBI for 6 months after MSC transplantation. Mahmood *et al.* [16,17] first investigated the effect of MSC on a TBI animal model and Bang *et al.* [11] reported the first autologous MSC transplantation in stroke patients. Both groups delivered MSC through intravenous injection. The MSC number in the injured area was proportionally related to the administrated cell number until the optimal amount was reached. The potential therapeutic benefit of MSC is presumably determined by its homing efficiency to the injured area. In this study, the combined procedure rendered MSC more likely to be exposed to the injured area, thereby enhancing the homing efficiency. This was also observed in a TBI rat model using carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled MSC (data not shown). The present study was designed as a safety and feasibility trial for the combined MSC delivery method to be used on TBI patients, therefore no control group was involved.

The functional improvement can probably be attributed to MSC homing to the injured area. Although the ultimate success or failure of cell therapy will rest on its ability to show clinical efficacy rather than the input mechanism, the variety of evidence from clinical and animal studies has indicated that MSC's direct differentiation and indirect effect through its secretion play an important role in promoting tissue recovery. By using green fluorescence protein (GFP) labeling and Y-chromosomal staining techniques, Mezey *et al.* [1,18] discovered new neurons in the brain generated from transplanted BM cells in studied mice and leukemic patients. Recent studies from Chopp's group [5,17,19,20] have suggested that the improvement of neuronal function is mainly mediated by local MSC-released growth and trophic factors. These factors promote synaptogenesis, angiogenesis and neurogenesis through endogenous neuron stem cell development. Further well-controlled studies are required to clarify the possible mechanisms.

In conclusion, autologous MSC was delivered to TBI patients by direct application followed by intravenous injection. The procedure proved feasible and safe during a 6-month follow-up period. The MSC homing efficacy was

presumably enhanced through this combined cell delivery procedure.

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