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# Reconstruction of the adenosine system by bone marrow-derived mesenchymal stem cell transplantation<sup>☆</sup>

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## Abstract

In the present study, we transplanted bone marrow-derived mesenchymal stem cells into the CA3 area of the hippocampus of chronic epilepsy rats kindled by lithium chloride-pilocarpine. Immunofluorescence and western blotting revealed an increase in adenosine A1 receptor expression and a decrease in adenosine A2a receptor expression in the brain tissues of epileptic rats 3 months after transplantation. Moreover, the imbalance in the A1 adenosine receptor/A2a adenosine receptor ratio was improved. Electroencephalograms showed that frequency and amplitude of spikes in the hippocampus and frontal lobe were reduced. These results suggested that mesenchymal stem cell transplantation can reconstruct the normal function of the adenosine system in the brain and greatly improve epileptiform discharges.

**Key Words:** bone marrow-derived mesenchymal stem cells; chronic epilepsy; cell transplantation; reconstruction; adenosine system; electroencephalogram; immunohistochemistry

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## INTRODUCTION

The dysfunction of the adenosine system in chronic epilepsy is characterized by the reduction of extracellular adenosine level, change of the density of adenosine receptors and over-expression of adenosine kinase<sup>[1]</sup>. This dysfunction contributes to the pathological mechanisms underlying epilepsy and uncontrolled seizures<sup>[2-4]</sup>. Therefore, reconstruction of adenosine system function is important for the treatment of refractory epilepsy. Many researchers have attempted to improve adenosine system dysfunction through the application of adenosine and its analogs, adenosine receptor agonists and antagonists<sup>[5-8]</sup>. However, most of them have failed because of a lack of adenosine receptors in the brain<sup>[9]</sup>. Moreover, these compounds were shown to have side effects, such as sedation and inhibition of the cardiovascular system<sup>[10-14]</sup>, which have restricted their clinical application. Therefore, restoration of the neural inhibitory and protective function of the adenosine system relies on reconstruction of the whole system—the ability of neurons to secrete adenosine and adenosine receptor density in astrocytes as a whole. Stem cell transplantation has been regarded

as an ideal method for such reconstruction because it is associated with minimal rejection in nervous tissue. Cell transplantation into the brain has several advantages. First, only a minimal amount of adenosine (20–50 ng/d) can inhibit epileptiform discharges<sup>[15]</sup>. Second, the concentration of adenosine secreted by transplanted cells is too low to activate the internal flow of adenosine conducted by adenosine transporters in the presynaptic membrane. Therefore, the adenosine level outside the cell membrane can be maintained at a higher level than normal<sup>[16]</sup>. Therefore, cell transplantation can provide anti-epileptic and neuroprotective effects through restoration of the adenosine system<sup>[17]</sup>. We hypothesized that transplanted stem cells could promote the functional and structural reconstruction of adenosine system, and tested this hypothesis in the present study.

## RESULTS

### Quantitative analysis of experimental animals

A total of 16 Wistar rats were equally and randomly divided into transplantation and model groups. An epilepsy model was induced by kindling with lithium chloride-pilocarpine. One month after

kindling, bone marrow-derived mesenchymal stem cells (MSCs) were transplanted into the CA3 area of the hippocampi of rats in the transplantation group, and the same amount of PBS was injected into the same area of rats in the model group. One rat in the model group died, but it was replaced with an additional one so that eight rats were included in the final analysis.

**Culture and identification of MSCs**

The cultured passage 1 cells exhibited a round appearance and suspended in the culture medium with strong refraction. The cells began to adhere to the culture flask and the shape changed to become flat and spindle-shaped at 5–7 days (Figure 1). CD45 is a surface marker of lymphocytes, and does not present on MSCs<sup>[18]</sup>. By contrast, CD90 and CD105 are markers of MSCs<sup>[18]</sup>. In the present study, the cultured cells were positive for CD90 and CD105 on the surface and negative for CD45, indicating that they were in fact MSCs (Figure 2).

**Adenosine receptor expression in the brains of epileptic rats after MSC transplantation**

Compared with the model group, the expression of the

adenosine A1 receptor in the temporal lobe and hippocampus was up-regulated ( $P < 0.05$ ,  $P < 0.01$ ) and expression of the adenosine A2a receptor in the thalamus was down-regulated ( $P < 0.05$ ) as detected by immunofluorescence and western blot methods; moreover, the imbalance in the adenosine receptor A1/A2a ratio was improved at 3 months after transplantation (Figures 3, 4 and Table 1).

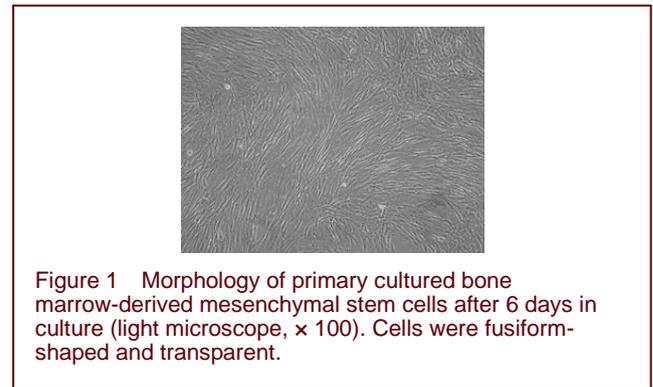


Figure 1 Morphology of primary cultured bone marrow-derived mesenchymal stem cells after 6 days in culture (light microscope, × 100). Cells were fusiform-shaped and transparent.

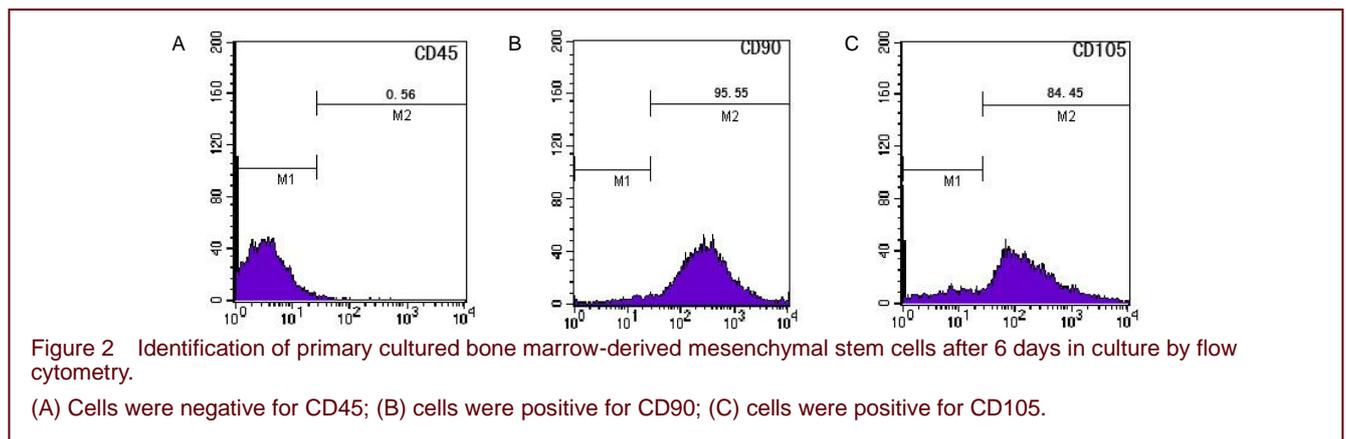


Figure 2 Identification of primary cultured bone marrow-derived mesenchymal stem cells after 6 days in culture by flow cytometry.

(A) Cells were negative for CD45; (B) cells were positive for CD90; (C) cells were positive for CD105.

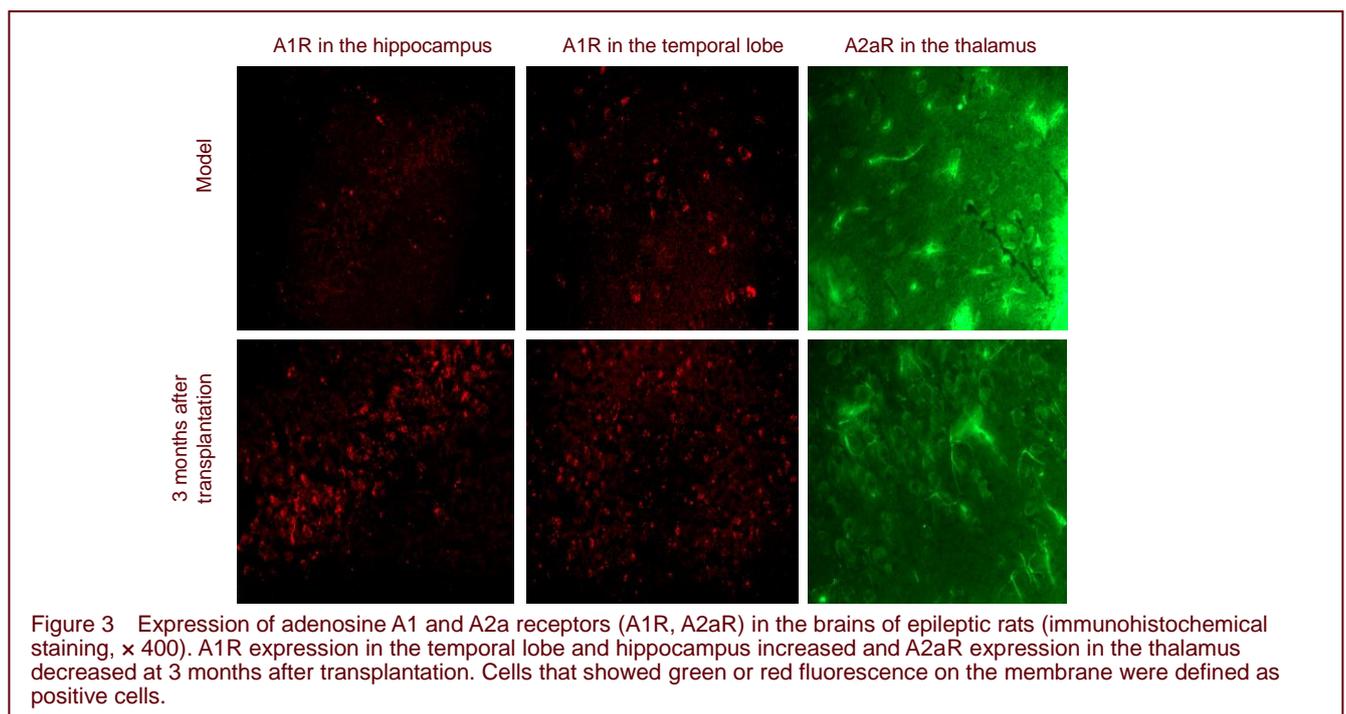


Figure 3 Expression of adenosine A1 and A2a receptors (A1R, A2aR) in the brains of epileptic rats (immunohistochemical staining, × 400). A1R expression in the temporal lobe and hippocampus increased and A2aR expression in the thalamus decreased at 3 months after transplantation. Cells that showed green or red fluorescence on the membrane were defined as positive cells.

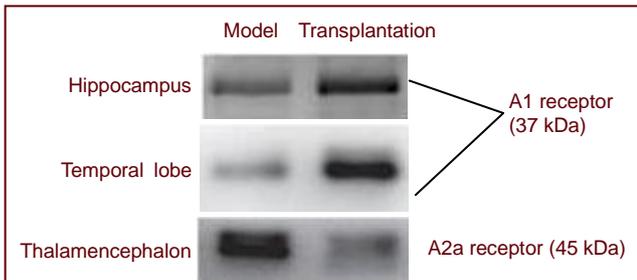


Figure 4 Expression of adenosine A1 receptor and A2a receptor in the brains of epileptic rats at 3 months after cell transplantation (western blot). The level of A1 receptor increased in the temporal lobe and hippocampus and that of A2a receptor decreased in the thalamus 3 months after transplantation.

Table 1 Numbers of adenosine A1 receptor- and A2a receptor-positive cells as observed by immunohistochemistry at 3 months after transplantation (cells/400-fold field of view)

Group	A1 receptor		A2a receptor in the thalamus
	Hippocampus	Temporal lobe	
Transplantation	46.18±6.19 <sup>a</sup>	58.37±4.23 <sup>b</sup>	27.64±4.71 <sup>b</sup>
Model	24.56±3.82	21.38±5.20	51.74±4.95

The number of A1 receptor-positive cells was increased in the temporal lobe and hippocampus and that of A2a receptor-positive cells decreased in the thalamus.

Data are expressed as means ± SD. Comparison of data between two groups was performed using one-way analysis of variance. <sup>a</sup> $P < 0.01$ , <sup>b</sup> $P < 0.05$ , vs. model group.

### Influence of MSC transplantation on epileptic discharges

Before transplantation, the spike discharge frequency was  $19.65 \pm 2.18$  per 29 cm and the amplitude was  $297.43 \pm 25.37$  mV in the cortex and hippocampus. After MSC transplantation, the spike discharge frequency was reduced to  $7.48 \pm 0.91$  per 29 cm ( $P < 0.01$ ), and the amplitude reduced to  $86.27 \pm 7.72$  mV ( $P < 0.01$ ; Figure 5).

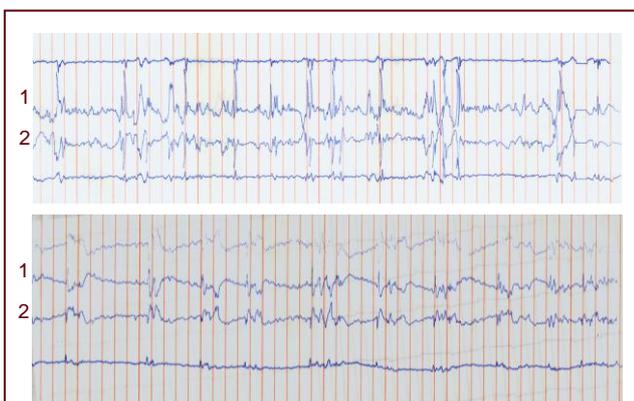


Figure 5 Alteration of electroencephalograms in epileptic rats. The amplitude and count of spike discharges were reduced. Lines 1 and 2 present results in the cortex and hippocampus in model rats, respectively (150 mV/cm). Upper: before transplantation; lower: 3 months after transplantation (1 mm = 25 V).

However, the count and amplitude of discharges in the model group remained unchanged after transplantation ( $P > 0.05$ ).

## DISCUSSION

Transplantation of MSCs has been shown to increase the amount of adenosine in the hippocampus of rats<sup>[19]</sup>. In the present study, at 3 months after transplantation, A1 adenosine receptor expression in the hippocampus and temporal lobe was significantly increased in epileptic rats, while A2a adenosine receptor expression in the thalamus was significantly decreased, indicating that MSC transplantation can improve the imbalance in adenosine receptors in the brain. Boison *et al*<sup>[20-21]</sup> transplanted embryonic stem cells into epileptic animals and concluded that the transplantation reduced both seizure frequency and electroencephalogram (EEG) discharges, consistent with the present results. Moreover, these authors indicated that transplanted stem cells can secrete adenosine, differentiate into functional neurons and connect with other neurological function cells. This ability of the cells makes them promising for use in anti-epileptic and neuroprotective therapies.

Because of the long-term stimulation of adenosine receptors in the brain by high levels of adenosine caused by seizures, the sensibility of these receptors gradually reduces, their expression changes and their density is disturbed<sup>[22-23]</sup>. That is, adenosine and its analogues can exaggerate the dysfunction of the adenosine system and seizures. However, does adenosine secreted by transplanted cells exaggerate adenosine system dysfunction? Gomes *et al*<sup>[17]</sup> suggested it did not, because (1) the improvement of seizure frequency was maintained for at least 8 weeks; (2) seizures in animals became more serious after application of selective A1 receptor antagonists, indicating that the inhibitory function mediated by adenosine A1 receptor is important in seizure control; and (3) with prolonged time, the transplanted cells gradually died and the secretion function gradually decreased (this was associated with an obvious increase in the frequency of seizures). All these findings show that adenosine secreted by transplanted cells can have an anti-epileptic function. In the present study, expression levels of both of the high-affinity adenosine receptors examined were improved 3 months after transplantation.

In summary, in the present study, we attempted to restore normal function to the adenosine system in the brain through the transplantation of MSCs. The amplitude and frequency of spike waves were significantly reduced and the imbalance in the expression of adenosine receptors was improved.

## MATERIALS AND METHODS

### Design

A randomized, controlled, animal study.

### Time and setting

The experiment was performed in the Neuroscience Laboratory of Tongji Hospital affiliated to Tongji Medical College, Huazhong University of Science and Technology, China from April 2007 to May 2008.

### Materials

Specific pathogen-free, male Wistar rats, aged 10–12 weeks, weighing 220–250 g, were purchased from the Animal Center of Tongji Medical College, Huazhong University of Science and Technology (license No. SYXK (E) 2004-0028). The animals were housed in groups of five under a continuous 12-hour dark/light cycle and allowed free access to water and food. All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China<sup>[24]</sup>.

### Methods

#### Culture and identification of MSCs

Wistar rats were sacrificed after anesthesia by diethyl ether and immersed in 75% alcohol for 8 minutes. The intact femur and tibia were separated, washed in iced phosphate buffered saline solution, and the muscle and bone membrane were removed. The ends of the femur and tibia were cut, and the marrow cavity was washed with phosphate buffered saline three times. The flushed fluid was centrifuged at 1 000 r/min for 10 minutes. The cells were suspended in complete medium (80% low-glucose Dulbecco's modified Eagle's medium/18% fetal calf serum/1% penicillin-streptomycin/1% glutamate/epidermal growth factor), plated in culture flasks at a density of  $5 \times 10^6$ /mL, and incubated in a saturated humidity incubator with 5% CO<sub>2</sub> at 37°C<sup>[25]</sup>. MSCs were identified by detecting the surface markers CD45, CD90 and CD105<sup>[18]</sup> using flow cytometry (BD, Franklin Lakes, NY, USA).

#### Preparation of epileptic model

The model was established according to previously described methods<sup>[26]</sup>. Briefly, animals were intraperitoneally injected with pilocarpine (50 mg/kg; Sigma, St. Louis, MO, USA) 18–24 hours after the intraperitoneal injection of lithium chloride (3 mmol/kg; Sigma). A majority of animals developed a limbic status epilepticus that was terminated by injection of diazepam (0.5–2.5 mg; Shihongkema Co., Ltd., Wuhan, China) 40 minutes after onset. Seizures that reached grade IV to V<sup>[27]</sup> were defined as fully kindled.

#### MSC transplantation into the hippocampus

Cultured and identified MSCs were trypsinized, suspended in PBS and transplanted at a density of  $2 \times 10^6$ /mL. Rats that had been kindled for 1 month were anesthetized by intraperitoneal injection of 6% chloral hydrate (0.5 mL/100 g). The rats were placed on a stereotaxic instrument (Morishige, Sr-5r, Japan) and the bregma and posterior fontanelle were maintained on the same line. The bregma was exposed and the right hippocampus was located (3.8 mm posterior to the bregma, 3.2 mm from the midline, 3.5 mm deep)<sup>[28]</sup>. The

skull was drilled with the pinhead of the injector until the dura mater was seen. A 2- $\mu$ L cell suspension was transplanted at a speed of 0.2  $\mu$ L/min for a total of 10 minutes using a microinjector (Micro-syringe, Chuangxintongheng Science and Technology Co., Ltd., Beijing). The needle was maintained for 10–15 minutes and slowly removed. The transplantation hole was closed with bone wax.

#### Immunofluorescence detection for adenosine receptor expression after transplantation

The rats were anesthetized with diethyl ether and sacrificed 3 months after transplantation. The brains were harvested immediately. Frozen sections of temporal lobe and thalamus (10 mm thick) were prepared to detect the expression of adenosine receptors by immunofluorescence. The primary antibodies used were rabbit anti-rat A1 receptor monoclonal antibody (1: 100; AbCam, Cambridge, UK) and mouse anti-rat A2a receptor monoclonal antibody (1: 100; Upstate, New York, NY, USA). Secondary antibodies were Cy3-conjugated goat anti-mouse polyclonal antibody (1: 300; Zhongshan Goldenbridge Biotechnology, Beijing, China) and fluorescein isothiocyanate-conjugated goat anti-rabbit polyclonal antibody (1: 300; Zhongshan Goldenbridge Biotechnology). Sections were incubated with primary antibodies for 2 hours and with secondary antibodies for 45 minutes. Cells that showed green or red fluorescence on the membrane were defined as positive cells. Two sections from each animal were selected and the number of positive cells in ten high-power fields of view ( $\times 400$ ) from one section was measured (Olympus, Tokyo, Japan).

#### Western blot detection for adenosine receptor expression after transplantation

Protein was extracted from temporal lobe and thalamus. After dilution in sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer, samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis between 120 V and 80 V, electrotransferred to polyvinylidene difluoride membranes at 200 mA for 60 minutes. Membranes were blocked at room temperature for 2 hours and incubated overnight at 4°C with antibodies against the rabbit anti-rat A1 receptor IgG (1: 1 000; AbCam) and mouse anti-rat A2a receptor IgG (1: 100; Upstate). After three washes in Tris-buffered saline containing Tween-20, each for 5 minutes, membranes were incubated with goat anti-rabbit/mouse IgG (1: 100; Boster, Wuhan, China) at room temperature for 60 minutes. After three washes in Tris-buffered saline containing Tween-20, each for 10 minutes, membranes were stained with diaminobenzidine (Zhongshan Goldenbridge Biotechnology).  $\beta$ -actin was used as an internal reference. Band intensities were analyzed using Image Quart Software (Gene genius, Syngene, Cambridge, UK). Relative density was calculated by dividing the intensity of adenosine receptor bands by that of  $\beta$ -actin bands. The intensity of each protein band was detected in

triplicate.

### EEG changes in rats after MSC transplantation

EEG recordings were performed before and 3 months after transplantation using an animal electroencephalograph (NT92-SH16, GM, Shanghai, China). Two electrodes were placed separately in the frontal lobe (2.0 mm anterior to the bregma, 2.0 mm from the midline and 0.5 mm deep) and in the CA3 of the hippocampus (2.0 mm posterior to the bregma, 3.2 mm from the midline and 3.5 mm deep). The other two were a groundwire and reference electrode. In the recorder, 2 mm amounted to 50 V and the running speed of paper was 15 mm/s. Recordings continued for 15 minutes. The average spike wave frequency and the amplitude of spike discharges were calculated. All the data were averaged.

### Statistical analysis

All data are expressed as mean  $\pm$  SD and analyzed using SPSS 11.5 software (SPSS, Chicago, IL, USA). Comparisons of data between two groups were performed by one-way analysis of variance. A value of  $P < 0.05$  was considered statistically different.

**Author contributions:** Huicong Kang designed the study, conducted the experiments, analyzed the experimental data and wrote the manuscript. Suiqiang Zhu designed and authorized the study, and provided instructions for the study. Qi Hu, Xiaoyan Liu, Feng Xu and Xiang Li analyzed the experimental data. Yinhe Liu revised the manuscript.

**Conflicts of interest:** None declared.

**Ethical approval:** The experiment was approved by the Animal Ethics Committee of Tongji Hospital affiliated to Tongji Medical College, China.

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