Improvement of the pilocarpine epilepsy model in rat using bone marrow stromal cell therapy

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Objective: Temporal lobe epilepsy is one of the most common types of epilepsy. Cytotherapy was tried for several neurological disorders. In this investigation, bone marrow stromal cells (BMSCs) were used in order to evaluate the recovery of epileptic rats induced by pilocarpine.

Methods: The rats were divided into four groups: a negative control, treated groups, and epileptic rats treated with the vehicle only. The animals in the chronic phase were monitored for three weeks using Racine scale. BMSCs were labeled with BrdU and injected intravenously. After 6 weeks, the rats were killed and processed in paraffin as well as immunohistochemical techniques.

Results: The results of the behavioral test showed that the number of seizures significantly decreased in treatment groups. Histopathology of the tissues from the untreated rats showed cell death and neurophagia. The numerical density of neurons per area was significantly higher in the treated groups than in the untreated ones. BMSCs localized in the hippocampus of the treated animals.

Discussion: The results showed that there is structural and functional improvement in the epileptic rats treated with BMSCs.

Keywords: Epilepsy, Pilocarpine, Epileptogenesis, Convulsion, Bone marrow stromal cells

Introduction

Temporal lobe epilepsy (TLE) is among the most common form of epilepsies in adults. TLE is characterized clinically by progressive development of spontaneous recurrent seizures of temporal lobe origin. A seizure, a condition in which there is an abnormal and recurrent neuronal discharge, often occurs as part of an epileptic syndrome (a group of signs and symptoms that customarily occur together). Patients with TLE usually suffer from secondary generalized tonic–clonic status.¹,²

One to two percent of the world population suffers epilepsy, but its prevalence and incidence are dramatically higher among elderly persons (over 60 years old) and children (2–14 years old).³ It is reported that the minimal incidence of status epilepticus (SE) in the Caucasian population of industrialized countries is about 20 per 100 000 a year, which depends on age, ethnic background, and possibly, gender. The range of the case fatality rate is from 1.9 to 40% depending on age, etiology, and duration of the SE.⁴ In Europe, the prevalence of epilepsy was estimated to be 4.3–7.8 per 1000, which cost 15.5 billion Euros in 2004.⁵

There are several problems with the current therapy, such as drug resistance and the invasiveness of neurosurgical treatment.⁶ Many other approaches were investigated including cytotherapy,¹,⁷,⁸ suggesting the use of cell therapy for epilepsy. Several experiments evaluated cell therapy in epilepsy,⁹ such as embryonic hippocampal cells transplantation, which resulted in local and long-distance synaptic formation with survival of the transplant,⁷ and engraft of human neural stem cells was used as alternative source for transplantation in epileptic rats, which resulted in prevention of spontaneous recurrent seizures.¹ Similar findings were reported by other investigators.¹⁰

Another source for adult stem cells is the bone marrow stromal cells (BMSCs),¹¹ which are multipotent cells that replicate as undifferentiated cells with the potential of differentiating into other cells such as bones, cartilages, fats, tendons, and muscles.¹² They may also transdifferentiate into cells of different lineages, such as neuronal phenotypes.¹³ The
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The experimental use of these cells in neuronal injury treatment showed promising results, i.e. their transplantation in the rat model of stroke reduced functional deficits, while similar findings were reported in traumatic brain injury, spinal cord injury, and neurodegenerative diseases.

Rat pilocarpine model of epilepsy is the best known model for investigation because it resembles human epilepsy, where following the injection of pilocarpine, the animals developed cholinergic effects, seizures, and subsequent signs of chronic epilepsy.

In this investigation, BMSCs have been used in order to treat pilocarpine-induced epileptic rats. For assessing the response to the treatment, Racine scale was used as a behavioral test, moreover, histological, morphometric, and immunohistochemical techniques were applied in order to evaluate the engraftment of the BMSCs in the hippocampus of the epileptic rats.

Materials and Methods

BMSC culture

The autologous BMSCs were obtained from adult Sprague-Dawley male rats (12 weeks old, weighing 250–300 g). The fresh bone marrow was aseptically aspirated with 5 ml of alpha-MEM from the tibias and the femurs and transferred and placed in a 75 cm² flask for tissue culture with 12 ml alpha-MEM containing 20% fetal bovine serum. The cells were incubated at 37°C in 5% CO₂ for 3 days. The adherent cells were collected and subcultured, and the culture medium was replaced three times a week. The cells were labeled with BrdU (5 μM) after four passages for 48 hours, and they were checked for viability; 95% of the labeled cells were viable.

All of the experiments were performed in accordance with the Guideline of the Ethical Committee in the School of Medical Sciences at Tarbiat Modares University.

Animal model

The adult male Sprague-Dawley rats weighing 250–300 g (25 rats were divided into four different groups and a normal control one) were housed under controlled standard conditions (12-hour light–dark schedule, room temperature of 21–22°C, and 45–55% humidity) with food and water available ad libitum.

Methylscopolamine bromide (1 mg/kg s.c.) was administered in order to limit the peripheral effects of pilocarpine hydrochloride (350–400 mg/kg s.c.), it was injected after 30 minutes. One hour after the onset of the seizures, 2.5 mg/kg diazepam was injected into the epileptic rats. Within 5 minutes after the pilocarpine injection, the rats developed diarrhea and other signs of cholinergic stimulation. During the following 15–20 minutes, they exhibited head bobbing, scratching, chewing, and exploratory behavior. Pilocarpine treatment induced the following behavioral changes: akinesia, facial automatisms, and limbic seizures consisting of forelimb clonus with rearing, salivation, masticatory jaw movements, and falling. Recurrent seizures started around 30–60 min after the pilocarpine administration, and the animals were unresponsive to their environment. The initial acute insult was followed by a seizure-free phase (silent) and finally a chronic period characterized by the occurrence of spontaneous seizures; the chronic phase started 6–7 days after the first attack.

Animal groups

The rats were divided into four groups, each group consisting of five rats: a negative control group consisted of epileptic rats treated with phosphate buffer saline [phosphate-buffered saline (PBS) as vehicle], untreated epileptic rats, and two groups of epileptic rats treated with BMSCs 24 and 36 hours after the first seizure. Another group of normal rats was used as a control group.

Intravenous BMSC injection

A suspension of 3 × 10⁶ BrdU-labeled BMSCs in 500 μl PBS was injected by intravenous route 24 and 36 hours after the first seizure.

Behavioral assessment

The animals in the chronic phase were monitored via a video monitoring system for 3 weeks (day and night). Racine scale was used for evaluating the behavior of the treated and the untreated groups using the randomized blind method.

Histological assessment

Forty-two days after the first SE, the animals were anesthetized with ketamine (44 mg/kg) and xylazine (13 mg/kg), transcardially perfused with 20 ml heparinized saline, and then perfused with 4% paraformaldehyde in PBS (pH 7.4). The brain tissues were immersed in the same fixative for 24 hours. For each animal, one hemisphere of 10 different sections (10 μm thick) (2800–3800 μm distance from the bregma) was used for histological evaluation and counting, using hematoxylin–eosin and cresyl violet stains, respectively.

Morphometric analysis

The cell counting was done using the following formula:

\[
\text{Total number of cells} = n \times t / (t + d - 2b)
\]

where the counting was carried out at 20 × (b=0.36 μm), 40 × (b=0.28 μm), and 100 × (b=0.19 μm), t is the section thickness, d is the diameter of nucleus, and n is number of nuclei. The numerical density per area \(N_{(a)}\) of the intact neurons was obtained by counting the number of the neurons with central
nucleoli and Nissl's substance, while the sampling was carried out according to Aherne and Dunnell. 27 The total number of intact neurons was divided by the total area in $\mu m^2$. $N(a)$ of the intact neurons was calculated according to Taki and Nickerson, 28 while the percentages of neuronal reduction were calculated according to Bahadori et al. 29

Immunohistochemical study
Indirect immunoperoxidase evaluated the labeling of BMSCs using BrdU mouse anti-BrdU monoclonal antibody (Sigma), which was incubated for 24 hours at 4°C, then labeled with secondary polyclonal antibody conjugated with peroxidase (rabbit anti-mouse antibody conjugated with peroxidase; Sigma). Double-label immunofluorescence was used on cryostat frozen sections. The sections were incubated with mouse anti-BrdU monoclonal antibody (Sigma, St Louis, MO, USA) for 24 hours at 4°C, which was followed by incubation with anti-mouse IgG antibody conjugated with rhodamine (Sigma). The sections were washed with phosphate buffer saline, incubated with mouse anti-neurofilament 200 monoclonal antibody (Sigma) for 2 hours at 37°C, incubated with anti-mouse IgG antibody conjugated with FITC (Chemicon International, Temecula, CA, USA) and examined with axiophot fluorescence microscope (Carl Ziess, Oberkochen, Germany).

Statistical analysis
The data were analyzed with one-way ANOVA and Tukey’s test.

Results
The results of the bone marrow primary culture showed heterogeneous cell population, many of which were round with few adherent ones (Fig. 1A). After 8 or 10 days (passage 4), the BMSCs were homogenous and consisted of monolayer of spindle-shaped adherent cells (Fig. 1B). Following BrdU labeling, the cells were immunostained with anti-BrdU antibody and incubated with peroxidase-conjugated secondary antibody and reacted diaminobenzidine, and 95% of the cells were immunoreactive to BrdU (Fig. 1C).

Behavioral test
Within 5 minutes after the pilocarpine injection, the rats developed diarrhea and other signs of cholinergic stimulation. During the following 15–20 minutes, they exhibited head bobbing, scratching, chewing, and exploratory behavior. Recurrent seizures started around 40–50 minutes following the pilocarpine administration. The results of chronic phase monitoring (during 3 weeks) are presented in Fig. 2. There is a significant difference in the number of seizure attacks in the animals treated with BMSCs compared with those of the untreated group and the animals given the vehicle only (PBS). No significant difference was noticed between the animals treated at 24 and 36 hours after the first seizure.

Figure 1 Phase contrast micrographs (A and B) of bone marrow stromal cells (BMSCs) in primary culture (A) (after 48 hours: passage 1). The BMSCs had round shapes, while they have more uniform spindle shape after 4 passages (B) (scale bar = 50 $\mu$m). C represents immunostaining of the cultured BMSCs labeled with BrdU. The BMSCs were incubated with mouse anti-BrdU antibody and labeled with secondary antibody conjugated with peroxidase. The arrow shows a mitotic cell immunoreactive with BrdU, ‘empty arrowhead’ represents the cells at early phase of cell cycle with low-density of immnuoreactivity, and ‘solid arrowhead’ represents cells with high-density labeling with BrdU (scale bar = 20 $\mu$m).

Figure 2 A histogram represents the response of epileptic rats to bone marrow stromal cell (BMSC) treatment and the number of attacks in 3 weeks for the untreated group ‘control’ (the white column), epileptic animals treated with vehicle (PBS), and the animals treated with BMSCs 24 and 36 hours after the first seizure. The number of seizure attacks in the animals treated with BMSCs after 24 hours of the first attack is significantly lower than those of the untreated group and the negative control group (the epileptic rats injected with vehicle: PBS) ($P<0.0005$ in both groups); similar findings were noticed in the 36 hrs group ($P<0.0005$ in both groups). No significant difference was noticed between the animals treated 24 and 36 hours after the first seizure ($P>0.1$); similar results were noticed between the untreated group and the negative control group (the epileptic rats injected with vehicle: PBS) ($P>0.1$).
Histological assessment

After 6 weeks from the first seizure, all of the animals were anesthetized and killed. One hemisphere of the brain was removed and processed in paraffin, and 10 μm sections were prepared and stained with cresyl violet in order to demonstrate Nissl’s substance. Figure 3 shows the cells in the CA2 region of the hippocampus stained with cresyl violet. At a low magnification, the photomicrographs of the untreated group show that there was thinning in the neuronal layer, where a single nucleus, few nuclear thickness, or no cellularity could be seen (lower left photomicrograph). The treated group had uniform neuronal layer thickness, which is comparable with the control group (Fig. 3, middle and right photomicrograph of the lower panel). At a higher magnification, the photomicrographs of the untreated group show that there was thinning in the neuronal layer, where a single nucleus, few nuclear thickness, or no cellularity could be seen (lower left photomicrograph). The treated group had uniform neuronal layer thickness, which is comparable with the control group (Fig. 3, middle and right photomicrograph of the lower panel). At a higher magnification, the photomicrographs of the untreated group show that there was thinning in the neuronal layer, where a single nucleus, few nuclear thickness, or no cellularity could be seen (lower left photomicrograph). The treated group had uniform neuronal layer thickness, which is comparable with the control group (Fig. 3, middle and right photomicrograph of the lower panel).

Morphometric study

The numerical densities per area $N_{(a)}$ of the intact neurons in different hippocampal regions including CA1, CA2, CA3, and dentate gyrus (DG) is presented in Fig. 5. $N_{(a)}$ in the untreated animals was significantly lower than those of the normal control and the treated groups in all regions ($P<0.05$). Also, $N_{(a)}$ was significantly lower in the treated animals compared with those of the normal controls in all regions (Fig. 5).

Figure 6 (upper histogram) shows $N_{(a)}$ in CA1 and CA3 in the normal group, the untreated animals, and the animals treated 24 and 36 hours after the first seizure attack. The result shows that $N_{(a)}$ was significantly lower in the untreated animals than those of the normal and the treated groups. There was a significant increase in $N_{(a)}$ in the treated groups compared with those of the untreated controls in both regions. The group treated after 24 hours of the first seizure showed no significant difference from the group treated after 36 hour, while values of $N_{(a)}$ in the treated and the untreated groups were significantly lower than that of the normal control group (Fig. 6, upper histogram). The percentage of neuronal reduction was significantly higher in the untreated...
animals than those of the treated and the normal groups in both regions; however, in the untreated control, CA1 showed higher reduction than CA3 while the reverse was noticed in both of the treatment groups.

Immunofluorescence staining

Figure 7 presents the results of double-label immunofluorescence of a tissue section from the treated group, where the migrated BMSCs labeled with BrdU in the injured hippocampus transdifferentiated into neurofilament 200 immunoreactive cells.

The percentages of the migrated BMSCs in the epileptic rats that received BrdU-labeled BMSCs after 24 and 36 hours of the first seizure were 15.8 and 13.2, respectively.

Discussion

Because of the side effects of the current pharmacological treatment protocols, the presence of resistance to pharmacological therapy and the invasiveness of surgical treatment, other approaches were suggested for the treatment of epilepsy including cell therapy.

In this investigation, BMSCs have been used for evaluating the recovery of epileptic rats induced by pilocarpine. This model induces cell death in all regions of the hippocampus and resembles human epilepsy. In this investigation, the animals treated with BMSCs for 24 and 36 hours after the first seizure showed significant reduction in the number of seizure attacks, which was consistent with the finding of other investigators who transplanted different types of cells for treating epilepsy in different animal models. Recently, Costa-Ferro et al. used bone
marrow mononuclear cells in treatment of epileptic rats and they concluded that there were structural and functional improvements following the treatment, which was consistent with findings of this investigation. One of the earliest attempts for cell therapy in epilepsy was done by Huber et al. using a genetically engineered fibroblast, where the cells were engineered to release adenosine encapsulated in semipermeable polymers, which resulted in reduction of seizure attacks. Similar investigation was done by Thompson using immortalized neurons which were engineered to produce GABA under the control of doxycycline, which resulted in suppression of seizures in the treated epileptic rats. Zaman and Shetty used fetal hippocampal CA3 cell grafts in rat with a kainate model of epilepsy pre-treated with fibroblast growth factor-2, which resulted in an increase in the survival of the treated rats. Similar results were reported by other investigators. Transplantation of human adult stem cells into the pilocarpine model of rat epilepsy resulted in their differentiation into neuronal phenotype and a decrease in spontaneous recurrent seizures. Several kinds of cells were pre-treated either with brain-derived neurotrophic factor, neurotrophin-3, and a caspase inhibitor or fibroblast growth factor and caspase inhibitor. It resulted in engraftment and survival of the transplant in the injured hippocampus. Neurons derived from embryonic stem cell transplantation in rats with chronic epilepsy were reported to improve seizure in rats and mice. Transplanting glial precursor cells derived from embryonic stem cells, which lacked both alleles of adenosine kinase (Adk<sup>−/−</sup>) and caused adenosine release, resulted in improvement of seizure in epileptic rats. Recently, allotransplantation of genetically engineered striatal GABAergic rat cell lines resulted in transient anticonvulsant effects, while others reported that GABA-producing cells transplant produced long-term alleviation of epilepsy. The most important aspect of the use of BMSCs is their autologous potential application for transplantation, thus reducing the risk of immunological rejection, with no ethical problem regarding their use.

This study shows significant decrease in the total neurons in the hippocampus and reduction in the thickness of hippocampal neuronal layer, which is characterized by neurophagia and increase in the neurons with pyknotic nuclei. These changes may be caused by neuronal damage as a result of neuronal necrosis; however, whether the damage is the result of local excitotoxic hyperactivity or leakage at the blood–brain barrier is unknown. In the pilocarpine model, some investigators revealed that pilocarpine could cause hypoxia, increase in free radical, lactate, and CO<sub>2</sub>, and a shift in pH resulting in blood–brain barrier breakage, Ca<sub>2+</sub> shift, and subsequent cell death.

Neurogenesis associated with epilepsy was reported in the adult mammalian forebrain subventricular zone, where the neural stem cells were reported to have the ability to proliferate, migrate, and differentiate in the hippocampal region of epileptic animals. In the pilocarpine model, prolonged seizure activity markedly increases neurogenesis in the DG of adult rats. This suggested the feasibility of cytotherapy for epileptic rats with adult stem cells such as neural stem cells or BMSCs. For example, administering neural stem cells intravenously in epileptic rats was reported to reduce pilocarpine-induced seizures. Several kinds of cells were experimentally used for transplantation in epileptic animal models including allograft and xenograft. These cells raised immunological and ethical concerns. On the other hand, BMSCs can be autologously transplanted and can replicate in undifferentiated forms, and have the potential to differentiate into other lineages. Moreover, they can express cytokines known to have roles in hemopoiesis, which have been reported to influence the differentiation of cells derived from bone marrow. Intravenously injected BMSCs have been found within liver, kidney, spleen, and bone marrow; however, most of the identified BMSCs encircled the microvessels in

![Figure 7 Photomicrographs show the double-label immunofluorescence of a tissue section from the treated group. The left photomicrograph shows a tissue section treated with mouse anti-neurofilament 200 monoclonal antibody followed by anti-mouse IgG antibody conjugated with FITC. The middle panel shows the same section treated with mouse anti-BrdU monoclonal antibody followed by anti-mouse IgG antibody conjugated with rhodamine. The right panel shows the merged images of the left and middle panels. The ‘arrow’ shows host neurons with their nuclei negative for anti-BrdU antibody, while ‘arrow head’ represents a neuron positive for both antibodies (scale bar=17 μm).]
these organs, while some cells were located in the parenchyma or congregated in the areas around the injured brain.\textsuperscript{60,61} In this study, the immunohistochemical technique demonstrated the migration and differentiation of intravenously injected BMSCs in the hippocampus of epileptic rats, which was consistent with other investigations concerning the migration of BMSCs in the injured region of the central nervous system, such as the spinal cord and brain tissues.\textsuperscript{60,62} Homing could be the possible explanation for BMSCs migration into the injured region due to the release of cytokines.\textsuperscript{63} Moreover, BMSCs can express different trophic factors and neurotrophins.\textsuperscript{64,65} This may explain the mechanism of BMSC transmigration by the cytokines released from the injured nervous tissue and resulted in increase in homing of the injected BMSCs\textsuperscript{66} and transdifferentiation of the transmigrated BMSCs into neuronal phenotype by the action of neurotrophins released by these transmigrated cells,\textsuperscript{59,60} possibly by autocrine or paracrine mechanisms by expressing the neurotrophins receptors too.\textsuperscript{67} This mechanism could lead to transdifferentiating BMSCs into BMSCs-derived neuronal phenotype.\textsuperscript{68} Others suggested that BMSCs fused with the indigenous stem cells of the injured nervous tissues resulting in BMSCs-derived neurons. An \textit{in vitro} study revealed the mechanism of differentiation of BMSCs into neuronal phenotype, where a green fluorescent protein expressing BMSC was cocultured with the PKH-26-labeled neurons resulted in cell population positive for both PKH-26 and green fluorescent protein.\textsuperscript{69} On the other hand, there is clear evidence for the fusion mechanism \textit{in vivo}.\textsuperscript{70} BMSCs were reported to fuse with adult neuronal stem cells resulting in colocalization of markers for the donor and the host cells in a single cell.\textsuperscript{71}

This suggests the possible use of cell therapy as another possible approach to the treatment of epilepsy and recommends further investigation in order to characterize the neurotransmitters of the transplant.

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