Intravenous Transplants of Human Adipose-Derived Stem Cell Protect the Rat Brain From Ischemia-Induced Damage

Bo Gong, MS,[*,†,1] Yongpin Dong, MS,[*,1] Chao He, MD, PhD,[*] Weiwei Jiang, MS, PhD,[*] Yi Shan, MD, PhD,[*] Betty Y. Zhou, MD, PhD,[‡] and Wenfang Li, MD, PhD[*]

Background: Survival following cardiac arrest (CA) and subsequent cardiopulmonary resuscitation (CPR), to a great extent, depends on brain damage. Adipose-derived stem cells (ADSCs), as a source of paracrine growth factors and the capacity of neural differentiation may reduce this brain damage. *Objective:* The purpose of this study is to evaluate the protection of ADSCs to brain damage following CPR. *Methods:* Rats were divided into 3 groups, sham, CA, and ADSCs group. Rats in sham group went through sham surgery. Rats in CA group went through CA, CPR, and injection PBS (phosphate buffer saline). Rats in ADSCs group went through CA, CPR, and intravenous injection of ADSCs. Rats in sham group were sacrificed immediately after operation. At 24, 72, and 168 hours after return of spontaneous circulation operation, rats in CA and ADSCs group were randomly selected and sacrificed. Brain damage was evaluated by using Neurological Deficit Scale (NDS) score, hippocampal pathology, serum level of S100β, and apoptosis ratio of hippocampal neurons. Protein of brain derived neurotrophic factor (BDNF) and IL-6 (interleukin-6) in the hippocampus were detected. *Results:* Compared with sham group, CA and ADSCs group showed a decrease in NDS score, an increased apoptosis ratio of hippocampal nerve cells, increased serum level of S100-β, and a significant increase in neuroprotective IL-6 and BDNF. In comparison to CA group, ADSCs group had a mild degree of brain damage and higher expression of IL-6 and BDNF. *Conclusions:* In the acute stage of cerebral injury following CA, ADSCs might improve the prognosis of brain damage by stimulating the expression of neuroprotective IL-6 and BDNF.

Key Words: Cardiopulmonary resuscitation—adipose-derived stem cells—brain damage—neuroprotective

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Introduction

Cardiac arrest (CA) has become a serious health problem which leads to high mortality and morbidity. In recent years, high quality resuscitation measures have led to higher rates of survival for patients with CA.[1] However, there are still quite a few surviving patients suffering from irreversible brain injury, which is also the main reason for aphasia, paralysis, vegetable state, and death following return of spontaneous circulation (ROSC).[2] Despite comprehensive measures such as mild hypothermia, medicine are adopted, however long-term survival rates of patients following ROSC remains low.[3] In china, only about 1% patients survival to discharge from hospital with good neurological outcome.[4] Reducing neuronal

From the *Department of Emergency, Changzheng Hospital, The Second Military Medical University, Shanghai, PR China; †Deparment of emergency, Hankou Branch of Central Theater General Hospital, Wuhan, PR China; and ‡Shenzhen Alpha-biotechnology Inc. Shenzhen, PR China.

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Address correspondence to Betty Y. Zhou, MD, PhD, Shenzhen Alpha-biotechnology Inc. Shenzhen, PR China. Wenfang Li, MD, PhD, Department of Emergency, Changzheng Hospital, The Second Military Medical University, Shanghai, PR China. E-mails: 18926064092@163.com, liwenfangsh@163.com.

[1]These authors contributed equally to this work.

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damage and promoting neurological recovery remain to be a critical problem in modern medicine.

Due to multipotent differentiation potential and specific surface antigen expression, bone marrow derived mesenchymal stem cells have drawn the attention of researchers and become a standard in the field of regenerative medicine since discovered by Friedenstein in 1960s. By now, mesenchymal stem cells (MSCs) have been successfully isolated from different tissue sources including periosteum, synovium, skeletal muscle, skin, peripheral blood, adipose tissue, bone marrow, and umbilical cord.[5] Nevertheless, bone marrow aspiration is a painful procedure, the numbers of cells acquired are usually low, proliferation and differentiation capacity of BMSCs (bone marrow mesenchymal stem cells) declines with donor age.[6,7] In recent years, more and more attention has been put on Adipose-derived stem cells (ADSCs), with similar differentiation ability and cell surface expression pattern to bone marrow derived mesenchymal stem cells. Fat tissue has abundant resource in human body. ADSCs are easy to acquire by less invasion methods from fat tissue and easily be cultured in standard culture conditions,[8] more important proliferation and differentiation capacity of ADSCs is remarkably stable and hardly influenced by donor age.[9,10] Such unique advantages make the ADSCs an attractive tool in clinical application.

Recent researches on animals show that ADSCs can promote neuronal regeneration, protect damaged neurons, decrease neuronal apoptosis, and promote the recovery of neurological function. For now, most studies are based on the middle cerebral artery occlusion model,[11-13] which is a focal cerebral ischemic model. Compared with middle cerebral artery occlusion, global brain injury following ROSC is different in both pathology and physiology, and more complex. The purpose of this study is to evaluate the therapeutic effect of ADSCs on the protection of ischemia/reperfusion brain damage following cardiopulmonary resuscitation (CPR).

Material and Methods

Preparation of ADSCs

ADSCs were provided by Shanghai Shenbei Biological Technology Co. Preparation procedure followed chemistry, manufacturing, and controls of Steminent Biotherapeutics Technology Co. More than 100 mL adipose tissue was extracted from healthy volunteer by liposuction; under sterile conditions, adipose tissue was minced, rinsed repeatedly with PBS solutions, digested with type I and II collagenase digestion, then the culture was centrifuged to remove the supernatant, rinsed repeatedly with PBS solutions and filtered to obtain cells. Cells were cultured with culture medium (DMEMF/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) with 10% FBS (Fetal Bovine Serum)), and passaged when the cell fusion was over 80% of the flask. Phenotype identification of cells passaged 3 generations was performed using flow cytometry with BD Stemflow hMSC analysis kit (bd biosciences). Osteogenesis differentiation of ADSCs passaged 6 generations was induced by Thermo StemPro osteogenesis differentiation kit (Thermo Fisher Scientific), and stained with Alizarin Red S solution (Sigma-Aldrich). Chondrogenesis differentiation was induced by Thermo StemPro chondrogenesis differentiation kit (Thermo Fisher Scientific), stained with Alcian blue solution (Sigma-Aldrich,). Adipogenesis differentiation was induced by Thermo StemPro adipogenesis differentiation kit (Thermo Fisher Scientific), stained with Oil-Red O solution (Sigma-Aldrich). Cells were passaged 10 generations were prepared for transplantation.

Experimental Animals

Healthy Sprague-Dawley (SD) rats weighting 350-450 g were provided by the laboratory animal center of the Second Military Medical University. Rats were housed at 20°C-24°C, with 50%-65% humidity, and were allowed free access to water and feeds. The study was approved by the Animal Ethics Committee of the Second Military Medical University.

Preparation of Rat Model of CA/Cardiopulmonary resuscitation

Healthy male SD rats were fed only water for 12 hours before operation. Animals were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg), additional doses were given at a speed of 10 mg/kg per hour. Furs on the neck, chest, and right groin areas were removed by an animal shaver. The rats were fixed on the operating area in supine position. Oral tracheal intubation was performed and the tube was fixed to the lip. After disinfection and dissection, the right femoral artery was exposed, a PE-50 catheter was inserted to the thoracic aorta along the femoral artery. The catheter was fixed with suture and connected to the pressure sensor of a multichannel physiological parameter instrument (Alcott Biotech Co. Ltd., China) for continuous monitoring of arterial pressure. Another PE-50 catheter was inserted to the inferior vena cava along the femoral vein for intravenous injection. Metal needles were inserted subcutaneously in the limbs and connected to the machine mentioned above for ECG (electrocardiogram) (lead II) monitoring. A temperature probe was inserted into the anus of the rats at a depth of 3 cm for continuous monitoring of temperature. Following surgery, baseline physiological parameters were recorded. CA was induced by clipping the tracheal catheter. The criteria of CA are an arterial blood pressure without fluctuation and a mean arterial pressure falling to 25 mm Hg (about 4-5 minutes). Five minutes after CA, chest compression and mechanical ventilation started at the same time. Chest compressions were initiated with a frequency of 160 bpm, a depth of one-third of the anteroposterior diameter. If the ECG showed ventricular fibrillation during compression, 2 J of energy was given for defibrillation. The endotracheal tube was connected to the

ventilator (Alcott Biotech Co. Ltd., China), a tidal volume of .65 mL/100 g and a respiratory rate of 80 beats/minute. ROSC was defined as return of a supraventricular rhythm and MAP (mean artery pressure) achieving 60 mm Hg for 5 minutes. Mechanical ventilation, blood pressure, and ECG were continuously monitored for 2 additional hours. Recovery failure was considered if rats failed to achieve ROSC after 10 minutes of chest compressions.

On the first day after operation, rats were housed in cages individually, received 5% glucose saline injection artificial feeding every 6 hours, with free access to water and feeds. Three days after operation rats were allowed to return to the CA group.

Groups

Forty-two rats were randomly divided into 3 groups, sham group (n = 6), CA group (n = 18), and ADSCs group (n = 18).

Rats in sham group went through sham surgical. Rats were sacrificed immediately after operation, serum and hippocampus tissue were harvested.

Rats in CA group went through CA and CPR, received 1 mL PBS by intravenous injection at 1 hour after ROSC. Rats in ADSCs group went through CA and CPR, received 5×10^6 ADSCs cells in 1 mL PBS by intravenous transplantation at 1 hour after ROSC. In CA group and in ADSCs group, rats were both randomly divided into 3 subgroups (n = 6). One subgroup was sacrificed at 24 hours, 72 hours, and 168 hours after ROSC, respectively.

Neurological Deficit Scores in Rats

Before sacrifice, neurological function scores were recorded using Neurological Deficit Scale (NDS) Score[14] (Table1) as a standard. Scores were performed by laboratory personnel without knowledge of grouping. NDS score was used to evaluate brain function, with a range of 0 to 80 points, determined by factors such as brain stem function, cranial nerve reflex, motor function, sensory function, and simple act reflection. A lower score represents a more serious brain injury.

Table 1. *Neurological Deficit Scale*

(A) General behavioral deficit—Total score = 19			
Consciousness	Normal 10	Stuporous 5	Comatose 0
Arousal	Eyes open spontaneously 3	Eyes open to pain 1	No eye opening 0
Respiration	Normal 6	Abnormal 3	Absent 0
(B) Brain-stem function—Total score = 21			
Olfaction	Present 3		Absent 0
Vision	Present 3		Absent 0
Pupillary reflex	Present 3		Absent 0
Corneal reflex	Present 3		Absent 0
Startle reflex	Present 3		Absent 0
Whisker stimulation	Present 3		Absent 0
Swallowing	Present 3		Absent 0
(C) Motor assessment Total score = 6			
Left side strength	Normal 3	Stiff/weak 1	No movement 0
Right side strength	Normal 3	Stiff/weak 1	No movement 0
(D) Sensory assessment—Total score = 6			
Left side pain	Brisk withdrawal with pain 3	Weak 1	No withdrawal 0
Right side pain	Brisk withdrawal with pain 3	Weak 1	No withdrawal 0
(E) Motor behavior—Total score = 6			
Gait coordination	Normal 3	Abnormal 1	Absent 0
Balance on beam	Normal 3	Abnormal 1	Absent 0
(F) Behavior—Total score = 12			
Righting reflex	Normal 3	Abnormal 1	Absent 0
Negative geotaxis	Normal 3	Abnormal 1	Absent 0
Visual placing	Normal 3	Abnormal 1	Absent 0
Turning alley	Normal 3	Abnormal 1	Absent 0
(G) Seizures—Total score = 10			
Seizures	No seizure 10	Focal seizure 5	General seizure 0

Serum S100-β

Rats were sacrificed by intraperitoneal injection of pentobarbital (45 mg/kg). Three mL blood samples were collected via the left femoral vein, injected into a drying tube for 10 minutes, and then centrifuged at 1200 g for 15 minutes. The supernatant was placed into a centrifuge tube and maintained at −80°C, and S100-β level was analyzed by ELISA (enzyme-linked immuno sorbent assay).

Tissue Samples Processing

After collection of blood samples, the hippocampus was removed quickly, and the left hippocampus was put into 4% formaldehyde solution. Conventional dehydration, transparent, dip wax, and embedding were used to produce of hippocampal tissue paraffin section. The right hippocampus was placed in cryopreservation tubes and stored in liquid nitrogen.

HE (hematoxylin-eosin) staining was performed on the paraffin sections, and the hippocampus CA1 region was observed under microscope.

Ratio of hippocampal neuronal apoptosis: the paraffin sections of hippocampus tissue were stained by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining (Roche Applied Science). Paraffin sections were dewaxed, digested with proteinase K, incubated for 30 minutes at 37°C, and rinsed with PBS. TUNEL reaction mixture was added, incubated in a dark humid chamber at 37°C for 60 minutes, and rinsed with PBS. A DAB (diaminobenzidine) mixture was added; apoptotic nuclei shrinkage shows as brown, round, crescent, or irregular shaped. A section was randomly selected for each group and viewed at 400 × for taking 5 pictures. The number of apoptotic cells and the total number of cells were counted, and Image-Pro Plus 6.0 software was used to calculate the ratio of positive cells (numbers of positive cells/total numbers of cells) as the apoptosis rate.

BDNF and IL-6 detection: Western blot was used to detect BDNF and IL-6 in the hippocampus. Frozen homogenate hippocampus specimens were initially centrifuged for 5 minutes (4°C, 1500 g). Ten percent SDS (Sodium Dodecyl Sulfonate) was added, then extracted protein and transferred to membrane. The transferred membrane was blocked for 1 hour with .5% TBST-20 (Tris Buffered Saline with Tween) buffer (with 5% skim milk), then Anti-BDNF antibody (Abcam) or Anti-IL-6 antibody (Abcam), relative anti-β-Actin antibody (Abcam) was added and incubated overnight. The membrane was rinsed with TBST-20, then horseradish peroxidase coupled with secondary antibody was added and incubated. A final rinse was completed. Software was used for gel imaging. Optical density values were analyzed by using image pro plus 6.0.

Statistics

In all of the test results, count data are present as ± s. SPSS 21.0 statistical software was used for analysis of the comparison of the 2 variances and multiple samples average, parametric data at the same time point among groups were analyzed using LSD test, where P value less than .05 was statistically significant.

Results

ADSCs

Primary cells adhered to the dish bottom after 24 hours. Cells began to proliferate and gradually grew to form small colonies. Over time, ADSCs were densely distributed, had uniform spindle morphology, and grew in a vortex-like manner (Fig1a). Cell phenotype was examined by flow cytometry. The percentage of CD73 positive cells was 99.99%, respectively, of CD90 99.99%, and of CD105 96.88%, but CD34, CD45, CD11b, CD19, and HLA-DR were negative (.33%) (Fig1b).

After osteogenesis differentiation, cells were vigorous growth, displayed short spindle or irregular shaped morphology, mineralization nodules were observed at seventh day. At 28th day, mineralization nodules were stained as red spots by alizarin red, which showed red bone-like tissue structures. After chondrogenesis differentiation, the formation of round or oval nodules were observed, cells were stained blue by alcian blue at seventh day. After adipogenesis differentiation, cells displayed round shaped morphology, lipid droplets were observed in cytoplasm, with positive oil red O staining at 14th day (Fig1c-e).

Neurological Deficit Score

The NDS score in CA group and ADSCs group following CA was significantly lower than in sham group. ($P < .05$), and gradually increased with time. NDS score in ADSCs group was significantly higher than in CA group at each time point ($P < .05$; Fig 2a).

Serum S100-β

S100-β level in CA group and ADSCs was significantly higher than in sham group ($P < .01$), and reached a peak at 24 hours. The level of S100-β in ADSCs group was significantly lower at each time point compared with S100-β in CA group ($P < .05$; Fig 2b).

Hippocampal neuron apoptosis ratio

Rats in sham group showed a very small number of TUNEL-positive cells, while rats in groups CA and ADSCs showed a large number of TUNEL-positive cells after CA, which were significantly higher than sham group ($P < .01$). Ratio of hippocampal neuronal apoptosis in ADSCs group was significantly lower than in CA group ($P < .05$) at each time point (Fig 3a, b).

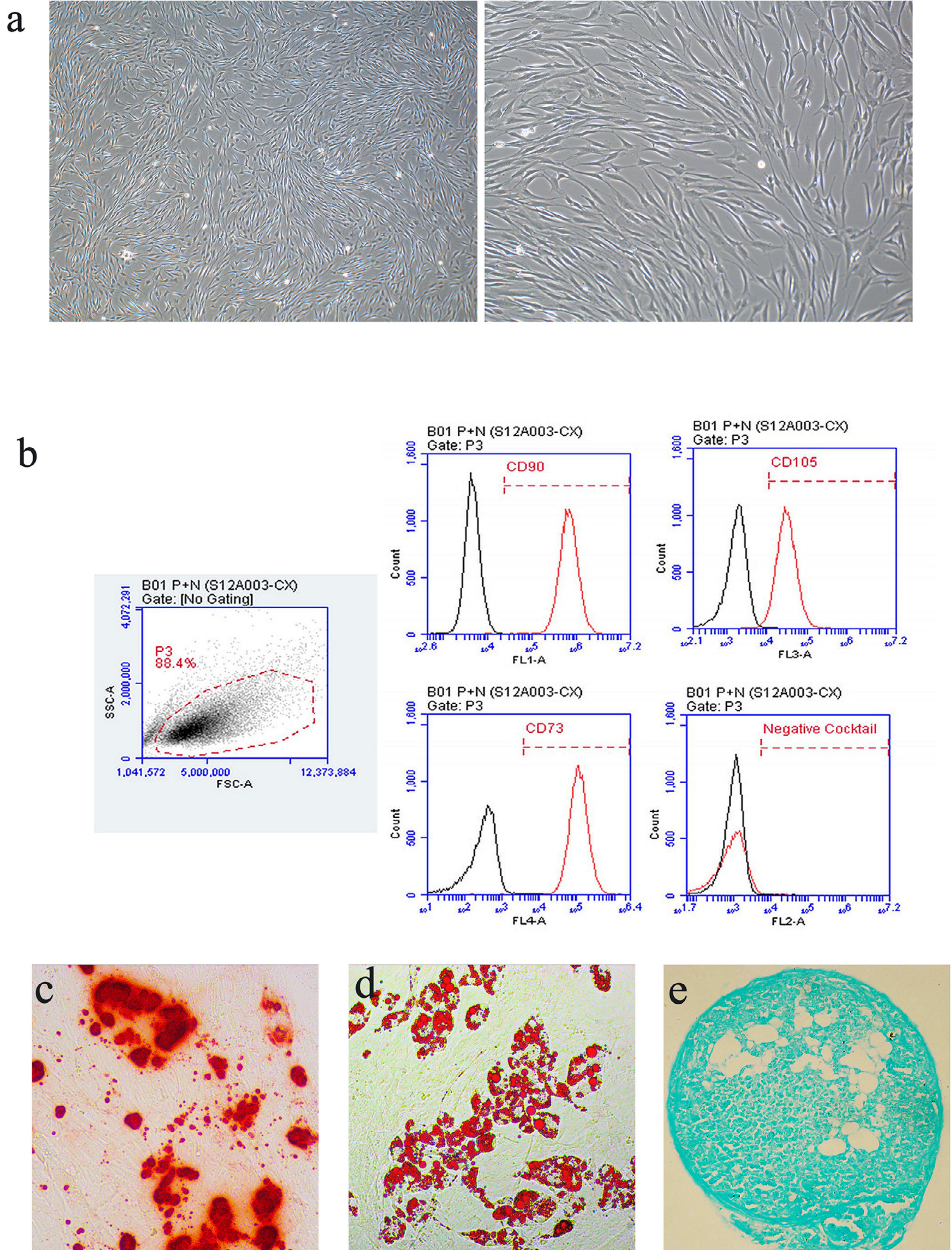

Figure 1. *The biological characteristics of ADSCs. (a) Morphology of ADSCs (40× and 100×). (b) CD73, CD90, and CD105 are positive on ADSCs, and CD34, CD45, CD11b, CD19, and HLA-DR are negative. (c) Osteogenesis differentiation and alizarin red staining at 28th day (100×). (d) Adipogenesis differentiation and oil red O staining at 14th day (100×). (e) Chondrogenesis differentiation and alcian blue staining at seventh day (100×). Abbreviations: ADSC, Adipose-derived stem cells; CA, Cardiac arrest. (Color version of figure is available online.)*

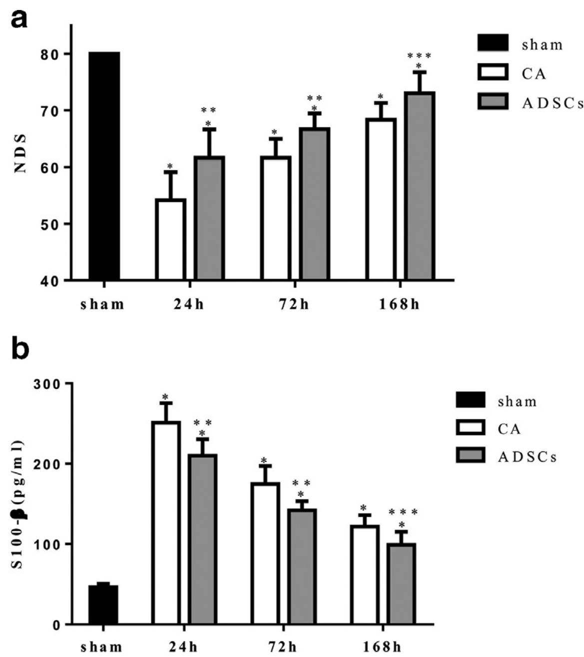

Figure 2. *The effect of ADSCs on nerve function. (a) Neurological deficit score, *P < .01 versus sham group, **P < .01 versus CA group at the same time point, and ***P = .01 versus CA group at the same time point. (b) Serum S100-β level, *P < .01 versus sham group, **P < .01 versus CA group at the same time point, and ***P < .05 group versus CA group at the same time point. Abbreviations: ADSC, Adipose-derived stem cells; CA, Cardiac arrest.*

Hippocampus BDNF

At 24 hours and 72 hours after ROSC, BDNF protein level in CA group and ADSCs group was significantly higher than in sham group($P < .01$), BDNF protein level in ADSCs group was significantly higher than in CA group($P < .01$). BDNF protein level decreased with time. At 168 hours after ROSC, BDNF protein level showed no significant difference among 3 groups (Fig 3d, e).

Hippocampus IL-6

Compared with sham group, IL-6 level of hippocampus in CA group and ADSCs group was increased ($P < .01$). At 24 hours and 72 hours after CPR, IL-6 level in ADSCs group was significantly higher than in CA group($P < .01$), at 168 hours after CPR, IL-6 level showed no significant difference between CA group and ADSCs group($P > .05$; Fig 3c, e).

Pathological Examination

Hippocampal tissue in sham group was determined to be morphologically normal. In CA group, necrosis of pyramidal cell numbers was decreased and proliferation of glial cells was observed in hippocampal CA1 region. ADSCs group also showed necrosis of pyramidal cells, however numbers of necrotic cells were significantly lower in ADSCs group, as compared with in CA group. In addition, nerve damage was significantly reduced in ADSCs group, as compared with in CA group (Fig 4).

In summary, rats in CA group and ADSCs group following CA, appeared varying degrees of neurological deficits. As compared with rats in sham group, rats in ADSCs group showed a mild degree of neurological injury, more IL-6 and BDNF were expressed.

Discussion

The main mechanism of brain injury is the whole brain ischemia/reperfusion injury.[15] Soon after blood flow reduction, various damage mechanisms are initiated, including free radical production, cell membrane depolarization, calcium overload, and acidosis, apoptotic pathways and inflammatory factors, continue in cascade amplification, resulting in neuronal necrosis or apoptosis.[15] Implementing effective cerebral resuscitation at the first few hours to reduce neuronal damage and promote neurological recovery remains to be a critical problem. We evaluated brain damage by NDS score, hippocampal pathology, serum level of S100-β, and apoptosis ratio of hippocampal neurons.

Clinically, serum S100-β level is used as an early biomarker to evaluate the degree of brain damage and to predict the prognosis of patients with neurological damage following CPR.[16,17] NDS score and ratio of neuron apoptosis in hippocampus were used to evaluate the degree of brain injury in rats. In this study, rats following CA appeared neurological deficits, as is demonstrated by increase of serum S100-β level and ratio of neuron apoptosis, decrease of NDS score, and pathological examination. Compared with rats with PBS injection, rats with ADSCs intravenous transplantation showed mild brain injury, which is manifested by lower S100-β level, less neuron apoptosis, and higher NDS score. The present study demonstrated that intravenous transplantation of ADSCs had neuroprotective effects in rats following CPR.

Reported studies have shown that ADSCs can alleviate neurological injury through a variety of mechanisms, including neural differentiation and neural substitution, paracrine function, immunoregulation, and endogenous neurogenesis. In the acute phase of brain injury, ADSCs probably play neuroprotective effects by its paracrine function which counteracts against inflammation and neuron apoptosis.[18] Transplantation ADSCs at 2-3 weeks after brain injury probably is superior in promoting endogenous neural recovery such as plasticity, angiogenesis, and neurogenesis, which are more intense at that time.[18] Therefore, we observe changes of IL-6 and BDNF affected by intravenous transplantation of ADSCs in hippocampal of rats.

IL-6 is originally recognized as a cytokine with both proinflammatory and anti-inflammatory effects, however its biological effects are not only limited to the immune system, but also involved in hematopoiesis, acute response phase, and the nervous system. In the normal brain, IL-6 is involved in neurogenesis and response of mature neurons and glial cells.[19,20] In the present study,

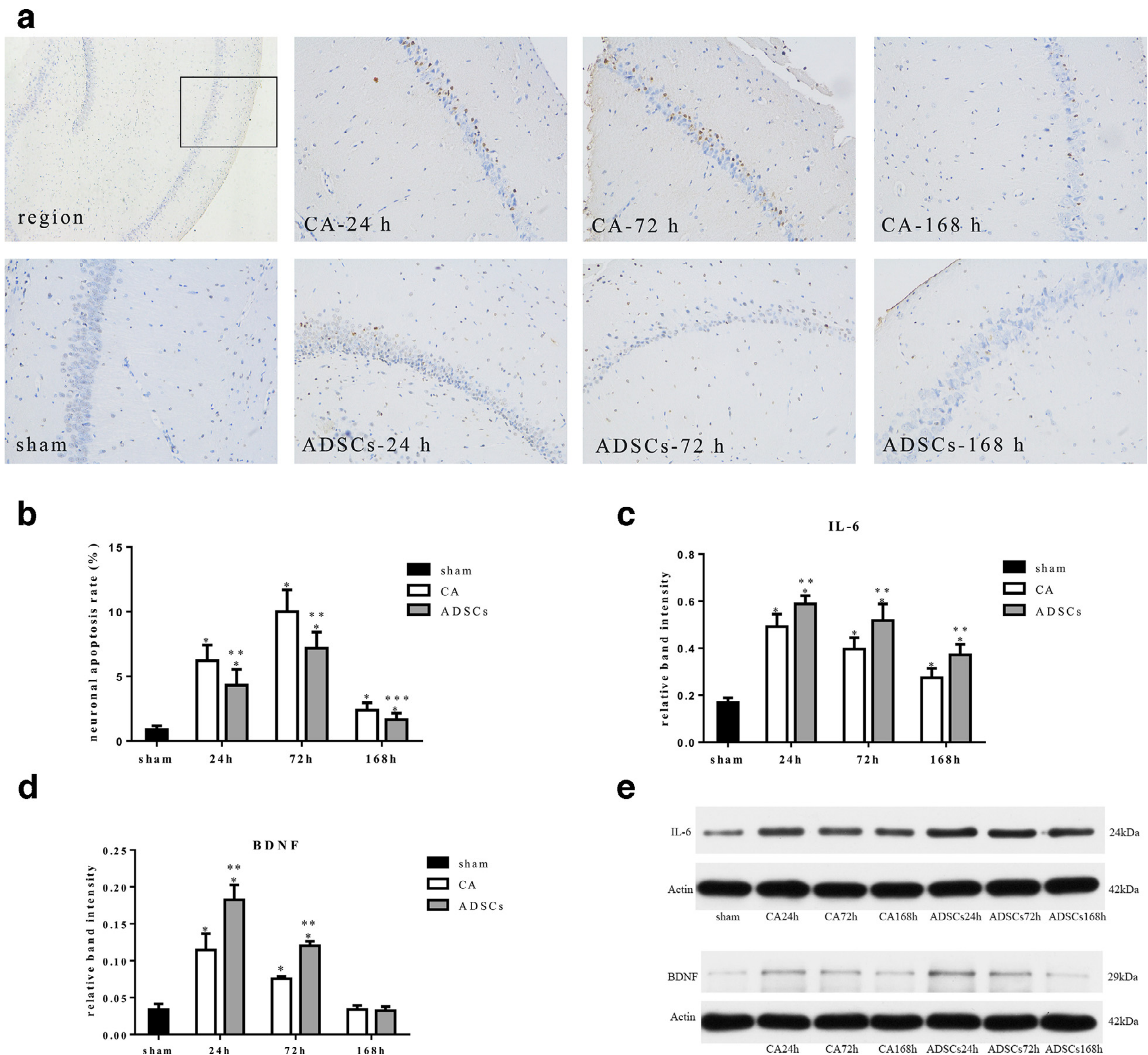

Figure 3. *Effects of ADSCs on apoptosis of nerve cells and neurotrophic factors. (a) TUNEL staining of hippocampus (200×). (b) ratio of hippocampal neuronal apoptosis, *P < .05 versus sham group, ** P < .01 versus CA group at the same time point, and *** P <.05 versus CA group at the same time point. (c) IL-6 level in hippocampus. (d) Relative band intensity of BDNF protein in hippocampus, *P < .01 versus sham group, **P < .01 versus CA group at the same time point. (e) IL-6 and BDNF in hippocampus by western blot. Abbreviations: ADSC, Adipose-derived stem cells; BDNF, brain derived neurotrophic factor; CA, Cardiac arrest.*

IL-6 expression in hippocampus of rats, intravenous transplantation ADSCs after ROSC, was significantly elevated. Over expression of IL-6 following MSCs transplantation was also observed in other studies.[21,22] It is believed that IL-6 plays a critical role in neuroprotection.[22,23] In brain injury model of rats, IL-6 binding with receptor IL-6R and glycoprotein 130 (gp130) can activate STAT3 pathway leading to increase the expression of antiapoptotic protein Bcl-2,[22] but decrease the expression level of proapoptotic protein Bax,[22] and reduce neuronal apoptosis. IL-6 also appeared to be involved in the regulation of cytokine secretion and blood-brain barrier integrity in cerebral ischemia.[23] However, further study is still required to understand the role of IL-6 in brain injury.

ADSCs can secrete a variety of trophic factors such as BDNF,[11,24] vascular endothelial growth factor, and nerve growth factor. These nutrients play an important role in promoting neural development and microvascular angiogenesis. BDNF is widely expressed in the central nervous system, which plays an important role in neural proliferation, differentiation, migration, and neuroprotection. When brain suffer ischemia reperfusion injury, BDNF protein can bind with TrkB receptor[25] and p75 receptor[26] to active several signaling pathways and to exert multiple protective roles, such as antiapoptosis, anti-inflammation, antineurotoxicity, and angiogenesis. In this study, BDNF protein expression in ADSCs group was significantly higher than in CA group, indicating that ADSCs can

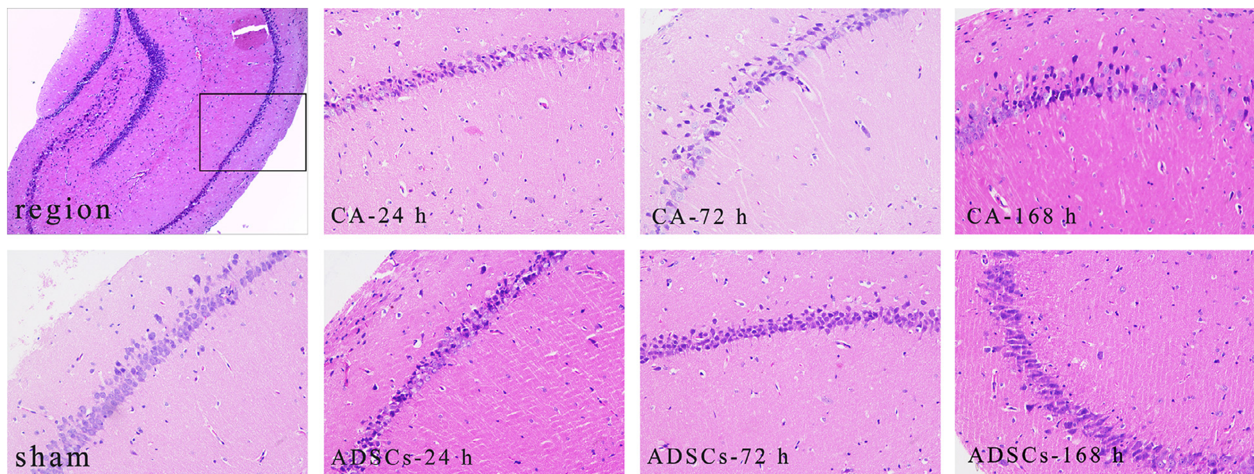

Figure 4. *Effect of ADSCs on pathological changes (200×). Hippocampal cell morphology was normal. CA group, numbers of pyramidal cells were markedly reduced. ADSCs group, numbers of pyramidal cells were slightly decreased. Morphological changes including chromatic agglutination (indicated by the black arrows), karyopyknosis, proliferation of glial cells (indicate by the red arrow) and vacuolization of neurons (indicate by the yellow arrow) could be observed under microscope. Abbreviations: ADSC, Adipose-derived stem cells; CA, Cardiac arrest. (Color version of figure is available online.)*

directly or indirectly upregulate BDNF protein expression. Studies showed ADSCs may promote BDNF expression via the activation of BDNF expression gating transcription factor[27] and TrkB signaling pathway.[13] In this study, BDNF level peaked at 24 hours, then gradually declined. At 168 hours after CPR, the level of BDNF in the hippocampus showed no significant difference among 3 groups, indicating that single-dose intravenous transplantation cannot maintain a high level of BDNF. Further study is required to determine whether multiple small doses transplantation can maintain BDNF at a higher level and have a better prognosis.

In conclusion, we demonstrated that delivering ADSCs by intravenous transplantation can decrease neurological deficits, improve neurological function for ischemia/reperfusion injury of brain following CPR. The neuroprotection function may be related with the expression of neuroprotective IL-6 and BDNF.

Conflict of Interest

No conflict of interest declared. All authors have read and approved the manuscript.

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