

Promoting the recovery of neurologic function and antagonizing cell apoptosis in ICH model rats by Induced Pluripotent Stem Cell Transplantation*

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Abstract: Intracerebral hemorrhage (ICH) is a major disease that seriously threatens human health; it features high fatality rate and disability rate and often leaves severe neurologic dysfunction even after recovery. The effect of induced pluripotent stem cells (iPSCs) transplantation on the recovery of the neurologic dysfunction of ICH is still uncertain. We prepared ICH rat models by injecting collagenase to the striatum of rats through stereotaxic apparatus, injected derived from ICH patients around the hematoma to evaluate the dynamic changes of neurologic function on day 1, 3, 7, 14 and 28 after ICH treatment by iPSCs transplantation with modified neurological severity score (mNSS) table, and studied the cell apoptosis changes around the hematoma. We found out that the neurological scores of rats were obviously improved and the apoptotic cells were significantly reduced compared with the model group. Our research results showed that iPSCs transplantation can effectively promote the recovery of neurologic function of ICH rats and antagonize cell apoptosis induced by encephalorrhagia.

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1. Introduction

After intracerebral hemorrhage (ICH), the secondary lesion is also the main cause of hemorrhagic brain injury in addition to the physical injury resulted from the hematoma's oppressing the surrounding brain tissues (Steiner and Bosel 2010). Histiocyte death resulted from secondary lesion after ICH has important clinical significance, and cell apoptosis as a gene-regulated manner of cell death may be one of the important parts (Felberg, Grotta et al. 2002). ICH is short of effective treatment means, and satisfactory curative effects are unable to be achieved even if the hematoma is timely eliminated.

Stem cell treatment is one of the possible treatment methods for ICH. Induced pluripotent stem cells (iPSCs) are a kind of pluripotent stem cell that can reprogram somatic cells to embryonic stem cell-like cells through its ectopic expression of some transcription factors (Lunn, Sakowski et al. 2011). As a totipotent cell, it can improve the neurologic functions of rats by means of neuron replacement, anti-inflammation, anti-apoptosis, angiogenesis promotion, myelin sheath and axon regeneration, and neuro-protection, but the specific mechanism is still not clear (Marchetto, Winner et al. 2010). In addition, it can

also avoid the ethical issues brought by embryonic stem cells, reduce immunological rejection, and provide possibilities of clinical application for autologous stem cell gene treatment (Lindvall and Kokaia 2011).

Cell apoptosis largely depends on the activation of cysteinyl aspartate-specific protease (caspase) family, in which caspase-3 is a key enzyme in mammalian cell apoptosis (Uccelli, Benvenuto et al. 2011). Recently, its effects in the neuron apoptosis have been observed by people, and it has also been confirmed by experiments that caspase-3 participates in various pathologic changes of brain injury, including transient cerebral ischemia, cerebral trauma and epilepsy and is also an important neuronal apoptosis effector. However, in this experiment, Oct4, Sox2, Klf4 and c-Myc genes were transformed into ICH patients' skin fibroblasts by lentiviral vector and further reprogrammed to iPSCs, TUNEL was used to conduct apoptotic cell counting and to observe the changes of caspase-3, and the possible mechanisms of ICH patient derived iPSCs transplantation improving neurologic function of ICH rats were discussed from the perspective of anti-apoptosis, which has not been reported in any literature yet.

2. Materials and Methods

2.1 cells

iPSCs came from our laboratory, and the cells were authorized by Ethics Committee of Zhengzhou University. Two days before recovery, we prepared a layer of mouse embryonic fibroblasts cells (MEF) feeders at 2.5×10^4 cells per well of 6-well plates using fibroblasts medium (Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% non-essential amino acids (NEAAs) (Invitrogen), and 100 IU/ml penicillin/streptomycin (Invitrogen)). 4-5 days, iPSCs clones were seen on MEF in hESC medium (DMEM/F12 supplemented with 20% KnockOut Serum Replacement (Invitrogen), 1 mM glutamine (Invitrogen), 1% NEAA, 0.1 mM β -mercaptoethanol (Sigma-Aldrich), and 4 ng/ml bFGF (Invitrogen)) and sub-cultured every 6-7 days or so. alkaline phosphatase test (AP Staining) was performed. Cells were fixed in 4% paraformaldehyde for 1-2 minutes. Aspirated and rinsed with 1 \times Rinse Buffer. Added stain solution (BufferA: BufferB: BufferC=100:38:28) enough to cover the well. The plate was incubated in dark at room temperature for 15 minutes. Then rinsed with 1 \times Rinse Buffer and observed under the microscope Cell.

2.2 Experimental animal grouping and modeling

Animal feeding and all relevant follow-up experimental procedures were authorized by Ethics Committee of Zhengzhou University and the Sprague-Dawley (SD) rats were provided by Henan Provincial Animal Center. 20 male rats (200-250g) were randomly divided into two groups: ten as a intracerebral hemorrhage PBS group (PBS group, after the models were identified as successful 24h after the operation, (10 μ L) PBS were injected by stereotaxic puncture, ten as a iPSCs intervention group (iPSCs group, after the models were identified as successful 24h after the operation, (1-2 $\times 10^5$ /uL) iPSCs were injected by stereotaxic puncture). iPSCs were incubated with 20 μ mol/L BrdU (Sigma-Aldrich) for 48 hours before transplantation. the rats were fixed on rat brain stereotaxic apparatus (Narishige SN-3, Tokyo, Japan) at a prone position, and by referring to the references (Jeong, Chu et al. 2003) (Fujiwara, Mandeville et al. 2011) and the Rat Brain Stereotaxic Graph of George Paxinos, needles were inserted perpendicularly for 6mm at 0.2mm back bregma and 3mm left, with the target point at the left corpus striatum. (0.5U/2u L) collagenase VII (Sigma-Aldrich) was injected slowly with a 10 μ L microsyringe, and the needle was retained for ten minutes and then withdrawn slowly. Then the wound surface was cleaned and the scalp was suture. mNSS scoring (Chen, Li et al.

2001; Lu, Chen et al. 2003) was performed 24h after the operation, and rats scored 8-12 in mNSS.

2.3 Behavioral Tests

mNSS scoring (including movement, sensation, balance and reflection) was adopted to evaluate the neurologic function deficit and recovery status of the rats, and each group was scored on day 1, 3, 7, 14 and 28 after operation

2.4 Immunohistochemical Staining

Samples of rat brain tissues were extracted in each group after 14 days of modeling; brain tissues of 3-5 rats were fixed with paraformaldehyde fixation, then dehydrated with gradient sucrose, frozen and cut into coronal slices (12 μ m thick). Each group of slices were conducted with immunofluorescence staining of brdu (rabbit anti-human as primary antibody and goat anti-rabbit, FITC conjugated conjugated as second antibody, Santa), and the immunohistochemical staining of caspase-3 (rabbit anti-rat as primary antibody and goat anti-rabbit as second antibody). TUNEL assay immunofluorescence detection of apoptosis (Roche).

The slices were re-warmed, fixed with 4% paraformaldehyde at RT for 5min, washed with PBS for 3 times and 5min/time, heated (92~98 $^{\circ}$ C) in 0.01mol/L pH6.0 citrate buffer solution for 30min for antigen retrieval and cooled naturally at RT for 30min; then the slices were washed with PBS for 5min for 3 times and sealed with 5% BSA at RT for 60min; subsequently, first antibody (1:100-1:500) was dropwise added on the slices and the slices were left at 4 $^{\circ}$ C over night and then washed with PBS for 5min for 3 times; then second antibody (1:500) was added at RT for 1h; afterwards, the slices were washed with PBS for 5min for 3 times; for immunohistochemistry, color was developed with DAB for 5min, and the slices were re-stained with haematoxylin and sealed.

2.5 Statistical Analysis

SPSS 13.0 statistical software was adopted to analyze the data and all measurement data were indicated in mean \pm standard deviation ($\bar{X} \pm S$), where, repeated measurement and analysis was conducted on mNSS data lines, Student t-test was conducted on the rest of the data. Data was considered statistically significant if $P < 0.05$.

3. Results

3.1 iPSCs preparations

iPSCs clones were seen on day 5 after recovery (Figure.1A). Then AP staining was performed and the stained cells presented modena, hence positive (Figure.1B).

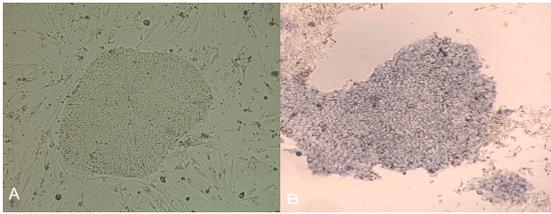


Figure 1. IPSCs clone (A). (100 ×) Alkaline phosphatase staining of iPSCs(B) (100 ×).

3.2 Behavioral Tests

After the rats were induced to ICH by stereotaxic collagenase injection, the rats showed contralateral hemiplegy among other neurologic function deficits soon. Symptoms and physical signs reached the peak 1~3d after the operation and then the rats recovered gradually. Compared with model group, the mNSS scoring of iPSCs group on day 7 after operation were different. The difference of mNSS scoring on day 14,28 was notable and significant ($P < 0.05$), (Figure.2).

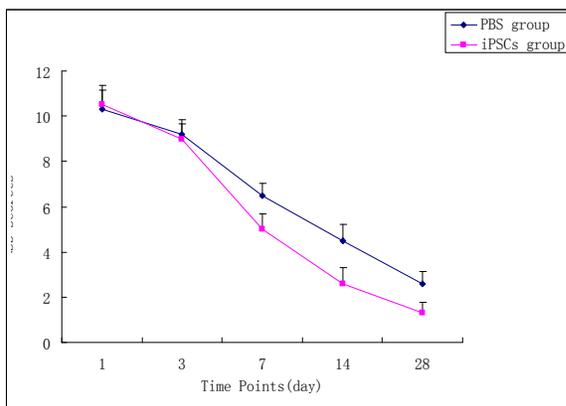


Figure 2. mNSS scoring at each time point for each group. Since day 14, scoring of iPSCs group is clearly superior to that of PBS group, ($p < 0.05$).

3.3 Brdu immunofluorescence staining

Under fluorescence microscope, it can be seen that Brdu immunofluorescence stained positive cells obviously gathered and spread around the hematoma(Figure.3A), and only few scattered on the opposite side of hematoma(Figure.3B).

3.4 TUNEL immunofluorescence staining

TUNEL staining showed that a great number of apoptotic cells existed around the nidus 14d after ICH, and the apoptotic cells were mainly located around the nidus in PBS group, while only few can be found on the opposite side. Besides, there were only few apoptotic cells seen in iPSCs group; the difference had statistical significance ($P < 0.05$). (Figure.3C-D,G).

3.5 Expression of caspase-3

caspase-3 was also expressed in each group and the expression level around the hematoma was higher. Cells were considered positive if the cytoplasm was stained brownish yellow granules; for negative cells, only the karyon was stained blue. The expression of caspase-3 in iPSCs group was relatively decreased compared with PBS group. The results showed that the difference of positive cell number between iPSCs group and PBS group had statistical significance ($P < 0.05$). (Figure.3E-F,G)

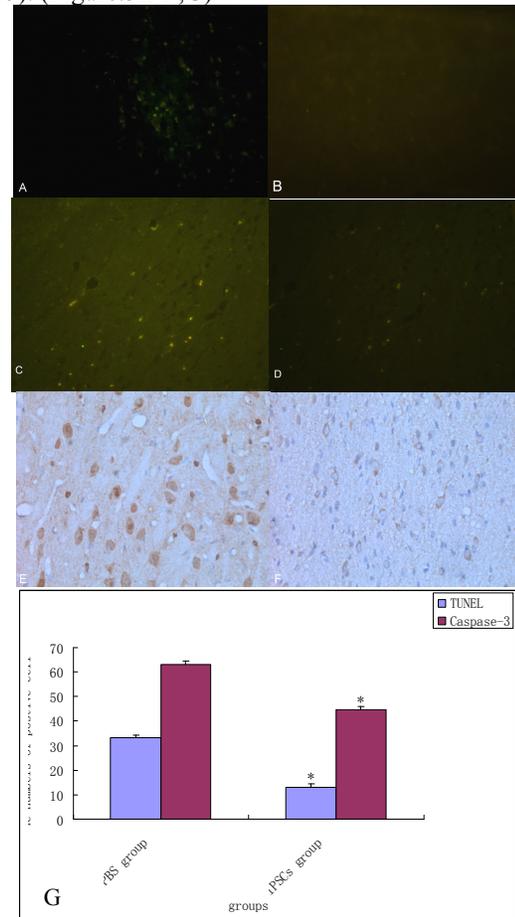


Figure 3. Brdu immunofluorescence stained positive cells obviously gathered and spread around the hematoma(A) (400 ×), and only few scattered on the opposite side of hematoma(B)(400 ×). TUNEL stained positive cells presented green globular shape. The apoptotic cells were mainly located around the nidus in PBS group(C)(200 ×). There were only few apoptotic cells seen in the iPSCs group(D)(200 ×). Numbers of apoptotic cells of iPSCs group(*) is higher than that of PBS group ($p < 0.05$) (G). Immunohistochemical staining of caspase-3 (E) is PBS group, and (F) is iPSCs group (400 ×). Numbers of positive cells of iPSCs group(*) is higher than that of PBS group ($p < 0.05$) (G).

4. Discussion

ICH has high incidence rate, high fatality rate and high disability rate. For most survivors, various degrees of neurological dysfunction may remain, yet at present, there is still no really effective treatment method for neurological impairment after ICH (Steiner and Bosel 2010). Therefore, in addition to further reinforcing the prevention research on ICH and the emergency measures in acute phase, seeking the effective treatment method to promote the recovery of damaged neurologic function is also the common objective of the numerous medical workers. Stem cells, especially with the appearance of iPSCs, have become the potential, possible effective method for ICH treatment (Lindvall and Kokaia 2011).

In this experiment, ICH models were successfully prepared by injecting collagenase to the striatum of rats through stereotaxis. After modeling, the symptoms and signs of neurologic deficit of rats were typical and could sustain for about 3 days, after which, there were various degrees of recovery. We transplanted iPSCs into the experimental ICH rat brains and observed the treatment effects. It was observed that transplanted iPSCs could survive in brain tissue and were mainly scattered around the hematoma through Brdu biomarker tracing, promoting that iPSCs could migrate towards the damaged parts of tissue. For iPSCs group, the scoring of neurologic function was superior to that of model group since day 7, and notably higher than the latter on day 14, 28. The difference had statistical significance. This showed from the point of behavioristics that iPSCs transplantation does have certain treatment effects in the ICH models.

After ICH, the hematoma not only triggers mass effect in the surrounding area, but also causes three pathologic changes: neuron and spongocyte death, vasogenic cerebral edema and damage of blood brain barrier (Lu, Chen et al. 2003). The exact mechanism relevant to pathologic changes of ICH is still not determined, and identifying the characteristics of the cell damage and death after ICH is helpful for researching the treatment measures of neurologic damage. During cell apoptosis, nuclear chromatin DNA double strands are broken into plenty of DNA fragments containing 3'-OH ends. Under the effect of terminal deoxynucleotidyl transferase (TDT) (Gong, Boulis et al. 2001), these fragments integrate with fluorescein-marked nucleotide and then the peroxidase-conjugated anti-fluorescein antibody, thus promoting the apoptotic cell staining, which is called terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, i.e. TUNEL staining (Kitaoka, Hua et al. 2002). We observed that the number of apoptotic cells in iPSCs group was obviously reduced compared with that of the PBS group by conducting TUNEL staining

research on ICH rat brain slices. The difference had statistical significance.

In recent studies, people have realized that Caspase-3 is a kind of cysteinyl aspartate-specific protease (caspase), and the proteolysis cascade it results selectively splits the substrate aspartate residue. Caspase-3 can activate the specific signal system and generate nuclear shrinking, DNA fragment formation and other apoptosis, eventually control the occurrence and development of apoptosis by splitting the protein kinase, nuclease and cytoskeleton (Huang, Xi et al. 2002). This experiment showed that Caspase-3 in iPSCs group is significantly reduced compared with that of PBS group. Besides, it is reported in some literature that Caspase-3 also plays a role in cell apoptosis by destroying the anti-apoptosis factor of cell (such as bcl-2) and the cell structure (such as structural protein actin, fodrin, lamin). Some scholars have proved with immunohistochemical double mark method that Caspase-3 and TUNEL staining coexist around the hemorrhagic nidus, and also confirmed that 95% are neurons and less than 5% are neuroglial cells. We adopt contiguous slices to separately conduct Caspase-3 immunohistochemistry and TUNEL staining, and both the results are basically consistent in terms of range and change of positive cells (Wu, Hua et al. 2002). Apoptosis mechanism may participate in some neuronal damages after ICH and the activation of caspase-3 has important regulation and control effect in the process of neuronal apoptosis after ICH.

In this experiment, we took the ICH model rats as the object of study and adopted iPSCs for transplantation. The results showed that iPSCs transplantation can effectively improve the neurologic dysfunction of ICH model rats. The mechanism may relate to apoptosis regulation and control. This study provides reliable preclinical theoretical basis for the further discussion of iPSCs transplantation, and eventually promotes its clinical application.

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