# Effect of bone marrow-derived mesenchymal stem cell transplantation on apoptosis and expression of P75NTR, TrkA in rats with intracerebral hemorrhage

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**Abstract**: Intracerebral hemorrhage (ICH)is a kind of serious nervous system disease of high incidence, morbidity and mortality. The major problem is that it leaves severe neurologic dysfunction even after recovery,threatens human health and lacks of effective treatment. Many researches on bone marrow-derived mesenchymal stem cells(BMSCs) transplantation have affirmed the improve effect on the recovery of the neurologic dysfunction of ICH,but the mechanism is still in exploration.So a rat model of ICH is established by stereotaxical injection of collagenase Ⅶ into the striatum and BMSCs are injected around the hematoma.We evaluate the change of neurologic function on day 1, 3, 7, 14 and 28 after ICH surgery by modified neurological severity score (mNSS) table and detect the change of expression of p75 neurotrophin receptor(P75NTR),tyrosine kinase A receptor(TrkA) and the cell apoptosis around the hematoma.It is found that the neurological scores of rats are obviously improved and the apoptotic cells are significantly reduced with lower P75NTR and higher TrkA expression compared with the ICH group. Our research shows that BMSCs transplantation could significantly promote the neurological function recovery in a rat model of ICH, which might be associated with its role in anti-apoptotic by down-regulating the expression of P75NTR and up-regulating the expression of TrkA in tissue around hematoma.

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**Key words：** bone marrow-derived mesenchymal stem cells (BMSCs), intracerebral hemorrhage (ICH), apoptosis, p75 neurotrophin receptor (P75NTR), tyrosine kinase A receptor (TrkA)

## 1.Introduction

When intracerebral hemorrhage(ICH) occurs, it can bring acute physical injury to the brain tissue surround the hematoma resulted from the hematoma's oppression (Steiner and Bosel. 2010).In addition,the secondary histiocyte death aggravates the hemorrhagic brain injury, accelerates the process and affects the prognosis of ICH. Therefore it has important theoretical and practical significance, and among which cellular apoptosis as a gene-regulated manner of programmed cell death has played an important role (Felberg RA et al.2002). So far ICH has been lacking of effective treatment and associated with a high morbidity and mortality.

Numbers of researches on bone marrow-derived mesenchymal stem cells (BMSCs) and a variety of disease models (including ICH) have affirmed their improving effect (Chen J et al. 2010, Seyfried DM et al.2011). What's more,they derived from the bone marrow, easy to acquire prepare and preserve, not involving ethics, hard to malignant transformation,no immune rejection reaction,can be self-donor transplanted,therefore they have broader prospects for clinical applications.As demonstrated they improve neurologic function by means of anti-apoptosis, neuro-protection, neurotrophic factor paracrine,and endogenous repair system activation (Uccelli, A., et al.2011) ,but the specific mechanism still remain unclear.

Nerve growth factor (NGF) can effectively influence the survival, apoptosis and differentiation of neurons.P75 neurotrophin receptor(P75NTR) and tyrosine kinase A (TrkA) receptor are both NGF receptors (Reichardt LF et al. 2006). It has been confirmed that neurotrophins facilitate cell survival and produce the nerve protection through activating TrkA receptors (Snider WD et al.1994). Meanwhile, P75NTR participates in the the apoptotic pathway of nerve cells in amyotrophic lateral sclerosis, Alzheimer's disease and spinal cord injury(Turner BJ et al.2003, Hashimoto Y et al.2004,Beattie et al.2002). Moreover the apoptosis mechanism it mediates also play an important role in the secondary injury of cerebral hemorrhage (G Bao et al 2010).

Research shows that BMSCs can reduce P75NTR expression in mice with experimental autoimmune encephalomyelitis (EAE) (Jing Zhang et al.2010). They can also reduce P75NTR and caspase 3

expressions in the oxygen-glucose deprived oligodendrocyte (OGD-OLGs), which leads to decreased oligodendrocyte apoptosis (Jing Zhang et al.2010). Whether the transplanted BMSCs play an anti-apoptotic role in improving neurological function of ICH rats by regulating the expression of these nerve growth factor receptors needs further study.So in this research,we use TUNEL to count apoptotic cell and observe the changes of the expression of P75NTR and TrkA to discuss the possible mechanism of BMSCs in improving neurologic function of ICH rats from the perspective of anti-apoptosis.

## 2. Materials and Methods

### 2.1 Derivation culture and identification of BMSCs

BMSCs are derived from an 8-week-aged rat according to the improved method that our laboratory described previously. All these following procedures are carried out in the central laboratory of Zhengzhou University and approved by the Ethics Committee of Zhengzhou University, Zhengzhou, China. Under aseptic conditions, we separate its femur and tibia, remove both epiphyseal ends and flush the marrow out to single cell suspension with 5ml Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM／F-12) DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAAs) (all from Invitrogen).Centrifuge the cell suspension at 1000 r/min for 5min abandoning the supernatant fluid,then sediment is inoculated with 5 ml medium described above in a 25 $cm^2$ cultivation of the bottle. The cells are incubated at 37°C and 5% CO2,the non-adherent of which is removed by changing the medium after 48 h.then the medium is changed every 3 day. The cells are passed with 0.25% trypsin / EDTA at a ratio of 1:3 until the cells achieve 80% confluence. Finally,the fifth generation cells are identified using flow cytometry to test the expression level of BMSC surface antigen CD44, CD105, CD34, CD45.

### 2.2 Experimental animal modeling and grouping

The Sprague-Dawley (SD) rats are provided by Henan Provincial Animal Center. Animal feeding and all relevant following experimental procedures are authorized by Ethics Committee of Zhengzhou University.The rats are fixed on rat brain stereotaxic apparatus (Narishige SN-3, Tokyo, Japan) at a prone position for stereotactic puncture,then micro-syringe needle is perpendicularly inserted into 6mm at the target of the left corpus striatum(0.2mm back bregma and 3mm left)referring to the references(Jeong, Chu et al. 2003) (Fujiwara, Mandeville et al. 2011) and the Rat Brain Stereotaxic Graph of George Paxinos. CollagenaseⅦ (0.5U/2ul) (Sigma-Aldrich) is injected at the speed of 0.5ul/min.Then needle is retained for ten minutes and then withdrawn slowly.Finally the wound surface is cleaned and the scalp is sutured. Bederson scoring is performed 24h after the operation, and rats scored 8-12 are adopted.30 adult male rats (200-250g) are randomly divided into three groups: ten as control (sham group, stereotactic puncture, not injection collagenase), ten as a intracerebral hemorrhage ICH group (ICH group, 24h after the models are made successfully,10ul ICH are injected by stereotaxic puncture), ten as a BMSCs intervention group (BMSCs group, 24h after the models are made successfully, BMSCs($1\text{-}2\times10^5$/uL) are injected by stereotaxic puncture. Before transplantation, the BMSCs are transfected with lentiviruses which carried the cDNAs of GFP 48 hours .)

### 2.3 Behavioral Tests

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

### 2.4 Immunohistochemical Staining and TUNEL Staining

14 days after modeling animals are anesthetized and perfused through the heart with ice-cold saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffe. Samples of rat brain in each group are extracted rapidly and then fixed with paraformaldehyde fixation at 4°C overnight. After that brain tissues are dehydrated with gradient sucrose, frozen, sectioned at a thickness of 10μm in the coronal plane and mounted on slides coated with poly-lysine. The immunohistochemical staining of P75NTR and TrkA. TUNEL immunofluorescence staining is used to detect apoptosis (Roche).

The slices are re-warmed at RT, fixed with 4% paraformaldehyde for 5min.Then it's time of antigen retrieval. The slices are washed with PBS for 5min 3 times,heated at 92~98℃ in citrate buffer solution (pH6.0) for 30min and cooled naturally at RT for 30min.Then the slices are washed as before and sealed by 5% normal goat serum (Boster) at RT for 60min, incubated by primary antibody (1:100-1:500, rabbit anti-rat, Santa) at 4℃ over night. Subsequently, the slices are washed with PBS again and incubated with biotinylated goat anti-rabbit IgG (1:200, ZSGB-Bio) for 2 h. Finally the slices are washed for immunohistochemistry using DBA for color development for 5min, re-stained with haematoxylin and sealed. The TUNEL staining is carried out according to the manual of Roche.

### 2.5 Statistical Analysis

SPSS 13.0 statistical software is used to analyze the data and all measurement data are indicated in mean ± standard deviation ( $\overline{X} \pm S$ ). Repeated measurement and analysis is conducted on mNSS data lines. Spearman correlation analysis is used for the

comparison of two variables.For other data, one-factor analysis of variance (ANOVA) is used.A P-value < 0.05 was considered statistically significant.

## 3. Results

### 3.1BMCSs preparations

The attached original generation BMSCs is like spindle, After P2 cells form a uniform fence and swirl shape. Flow cytometry is used to test BMSCs surface antigen marks, the positive rate of CD105 and CD44 is 99.2% and 99.6%, respectively, the negative rate of CD34 and CD45 is 100.0% and 99.8% respectively. After the success of the transfection, green fluorescence could be observed when the BMSCs were visualized under a fluorescence microscope due to the GFP. While it is the same with the BMSCs after they gather around the hematoma in brain.

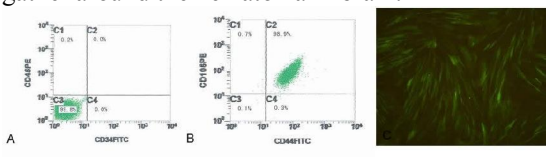

Figure 1. (A.B) Results of Flow cytometry; (C) BMSCs with GFP under fluorescence microscope

### 3.2 Behavioral Tests

After the rats are induced to ICH by stereotaxic collagenase injection,soon the rats show neurologic function deficits like contralateral hemiplegy and circle phenomenon. Symptoms and physical signs reach the peak 1~3d after the operation and then the rats recover gradually. The mNSS score of BMSCs group on day 7 after operation are lower compared with model group. The difference of mNSS score on day 14 is notable and significant (P＜0.05), (Figure.2) .

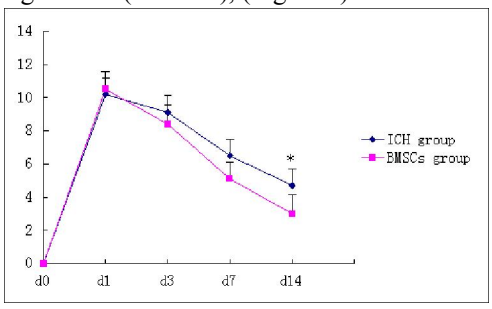


Figure 2. MNSS score of three groups at each time point. Score of BMSCs group on day 14 is clearly superior to that of ICH group, (p<0.05)(C).

### 3.3TUNEL immunofluorescence staining

TUNEL staining show that a great number of apoptotic cells existed around the nidus 14d after ICH, and the apoptotic cells are mainly located around the nidus in ICH group, while only few can be found on the opposite side. Besides, there are few apoptotic cells seen in BMSCs group; the difference has statistical significance (P<0.05). (Figure.3A~D)

### 3.4 Expression of P75NTR and TrkA

P75NTR and TrkA are expressed in all the three groups. Cells are considered immuno-histochemical positive if the cytoplasm is dyed brown or claybank granular; for negative cells, only the karyon is stained blue,without claybank granular.For P75NTR, there is only a few scattered positive cells in sham group,and positive cells of ICH group and BMSCs group are significantly increased. While The expression of P75NTR in BMSCs group is relatively reduced compared with ICH group. The results showed that the difference of positive cell number between BMSCs group and ICH group has statistical significance (P＜0.05). For TrkA there is a small amount of positive cells in both sham group and ICH group,compared with the two groups,the positive cells in BSMCs group are much more.The difference has statistical significance (P＜0.05). (Figure. 3.D~J)

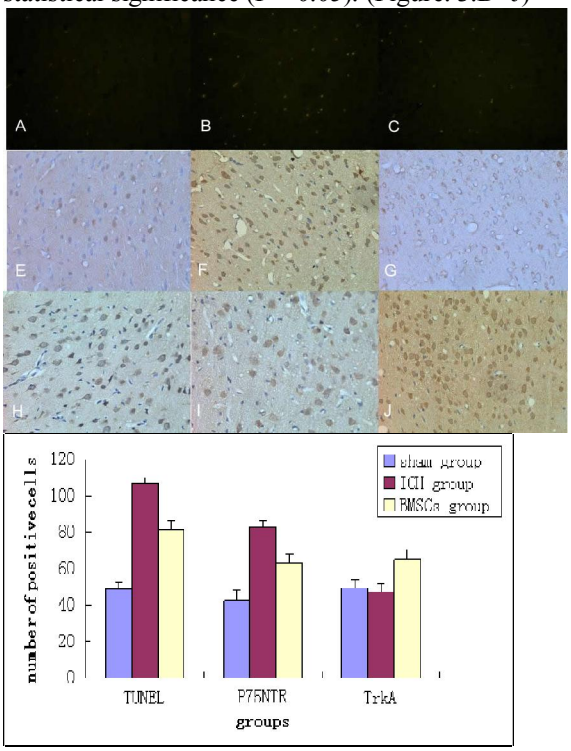


Figure 3. TUNEL positive cells present green globular shape. The apoptotic cells are located around the nidus in ICH group(A)(200 ×). (B.C) are TUNEL stain of BMSCs group and sham group. Numbers of positive cells of ICH group and BMSCs group(*) is more than that of sham group(p<0.05), while that of BMSCs goup is less than ICH group(p<0.05)(D). (E.F.G) are P75NTR immunohistochemical staining of ICH group BMSCs group and sham group(400×). Numbers of positive cells of BMSCs group and ICH group(*) is more than that of sham group(p<0.05),while that of BMSCs goup is less than ICH group(p<0.05) (D). (H.I.J) are TrkA immunohistochemical staining of ICH

group BMSCs group and sham group(400×).TrkA positive cell number of sham group and ICH group is close, the difference was not statistically significant (P = 0.117)(D), while that of BMSCs group is higher than model group and sham group, the difference was statistically significant (P < 0.05)(D)

**3.4The correlation of P75NTR and TUNEL positive cell**

Pearson correlation analysis is adopted to analysis the correlation of P75NTR and TUNEL staining positive cell number. Results show that there is statistical significance (r = 0.955, P < 0.000)

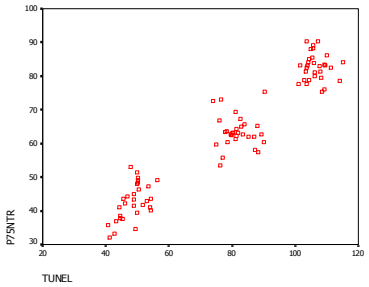


Figure. 4. P75NTR positive cell number and TUNEL staining positive cell number are positively correlated.

**4. Discussion**

ICH is associated with a high morbidity and mortality due to lack of effective therapy for neurological impairment.Most survivors remain various degrees of neurological dysfunction. (Steiner and Bosel. 2010). Therefore, besides enhancing prevention research and the emergency treatment in acute phase of ICH , an effective treatment to prevent the process of ICH and promote the recovery of damaged neurologic function in later period seems also urgent to numerous medical workers. As the development of stem cells,especial BMSCs,they have been proved to be a possible effective method for ICH treatment (Chen J et al.2010).

Our experiment results show that stereospecific injection of collagenase to the striatum of rats can induce ICH models successfully. After modeling, typical signs and symptoms of neurologic function defect of ICH rats appear soon and sustainable to 3 days or so,after that, they restore gradually to various degrees. For sham group ,there are no nerve function defect performance on each time point as control.We transplant BMSCs into the brain of ICH rat and observe the curative effects. It was obvious that transplanted BMSCs can survive in brain tissue around the hematoma promoting the eurologic function defect (Seyfried DM et al.2011).For BMSCs group, the scoring of neurologic function is superior to that of ICH group since day 7, and obvious lower than the latter on day 14. The difference has statistical significance. From the point of behavioristics,we can get the conclusion that BMSCs transplantation does have certain curative effects on ICH rats(Otero L et al. 2010).

After ICH, neurological function damage is a complicated multifactorial process,which include the size of the hematoma ,the severity of edema, the level of the calcium overload and the apoptosis of cells(Han N et al 2008).During cell apoptosis, the chromatin's DNA double strands are broken into fragments with 3'-OH ends which integrate with fluorescein-marked nucleotide and then the peroxidase-conjugated anti-fluorescein antibody detected by TUNEL staining.So we can observe that around the hematoma there are many apoptotic cells indicting that there are apoptosis after ICH leading to the neurological function defect.Meanwhile the number of apoptotic cells in BMSCs group is obviously reduced.The difference had statistical significance.It indicate that BMSCs may improve neurologic function by means of anti-apoptosis.

The number of apoptotic cells can directly influence the recovery of neurological function after ICH. The save of neuron surrounding hematoma largely depend on the hold back of the apoptosis start factor.In recent studies, people have focused on the NGF receptors including P75NTR and TrkA. Neurotrophins can facilitate cell survival and protect the nerve through activating TrkA.Meanwhile,P75NTR participates in the the apoptotic pathway of nerve in amyotrophic lateral sclerosis Alzheimer's disease, spinal cord injury and ICH.Knockout or blocking p75NTR signal can delay the disease progress and promote cell survival as reported(Turner BJ et al.2003).It is the same as our results.P75NTR signaling pathway can more effectively mediate apoptosis when TrkA don't express or express lowly.And it is also reported that BMSCs can reduce P75NTR expression in the oxygen-glucose deprived oligodendrocyte and mice with experimental autoimmune encephalomyelitis (EAE) (Jing Zhang et al.2010). In this experiment,the apoptotic cells around the hematoma decrease after the transplantation of BMSCs compared with ICH model rats, while the expression of P75NTR decreases significantly and the expression of TrkA increases significantly.

In this experiment, we produce the ICH model rat successfully as the object of study and transplant BMSCs into it. The results show that P75NTR plays an important role in the apoptosis of ICH leading to the neurologic dysfunction,while transplantation of BMSCs can effectively improve the neurologic dysfunction of ICH model rats through anti-apoptotic by down-regulating the expression of P75NTR and up-regulating the expression of TrkA.

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