• Basic Research •

Neuroprotective effects of BDNF and GDNF in intravitreally transplanted mesenchymal stem cells after optic nerve crush in mice

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Abstract

• AIM: To assess the neuro-protective effect of bone marrow mesenchymal stem cells (BMSCs) on retinal ganglion cells (RGCs) following optic nerve crush in mice.

• METHODS: C56BL/6J mice were treated with intravitreal injection of PBS, BMSCs, BDNF-interference BMSCs (BIM), and GDNF-interference BMSCs (GIM) following optic nerve crush, respectively. The number of surviving RGCs was determined by whole-mount retinas and frozen sections, while certain mRNA or protein was detected by q-PCR or ELISA, respectively.

• RESULTS: The density (cell number/mm^2) of RGCs was 410.77±56.70 in the retina 21d after optic nerve crush without any treatment, compared to 1351.39±195.97 in the normal control (P<0.05). RGCs in BMSCs treated eyes was 625.07±89.64/mm^2, significantly higher than that of no or PBS treatment (P<0.05). While RGCs was even less in the retina with intravitreal injection of BIM (354.07±39.77) and GIM (326.67±33.37) than that without treatment (P<0.05). BMSCs injection improved the internal BDNF expression in retinas.

• CONCLUSION: Optic nerve crush caused rust loss of RGCs and intravitreally transplanted BMSCs at some extent protected RGCs from death. The effect of BMSCs and level of BDNF in retinas are both related to BDNF and GDNF expression in BMSCs.

• KEYWORDS: optic nerve crush; retinal ganglion cells; stem cells; brain-derived neurotrophic factor; glial cell line-derived neurotrophic factor

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INTRODUCTION

Evidences have shown that the number of patients who are suffering from optic nerve related diseases, such as glaucoma and traumatic optic nerve injury, as one of the leading causes of vision disability worldwide, is increasing[1-2]. Optic nerve is part of central nervous system and formed by axons of retinal ganglion cells (RGCs), which are the only neurons to transfer the photoelectric information of retina to brain. Optic nerve injury might cause irreversible death of RGCs, resulting in vision loss. However, this kind of disease is lack of effective treatment[3-4], and the exploring of new ways are essential. Animal model of optic nerve crush (ONC) might lead to significant loss of RGCs, similar to the clinic situation of optic nerve related diseases, and can be used to observe the pathophysiological changes and prognosis of these diseases.

According to previous studies, the death of RGCs was mainly due to the changing of their micro environment[5-7], including the decrease of neurotrophic factors and increase of neurite growth inhibitory molecules. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3) were the main factors. Exogenous supplement of these neurotrophic factors might to some extent improve the survival of RGCs[8-10], but not stable [11]. It was found that[12] intravitreal injection of GDNF microsphere had better protective effect of RGCs than injection of GDNF itself. Stem cell transplantation, as a new method used for the treatment of nervous system diseases, has well been documented[13-14]. Researchers found that[14-19], intravitreal transplantation of bone mesenchymal stem cells (BMSCs) could significantly reduce the death of RGCs. The mechanisms were autocrine of neurotrophic factors by BMSCs[18,20-22], gene modification[23-25], immunoregulation[26-29] or differentiation into neurons[14,30-31]. In this study, we investigated the effects of BMSCs on the survival of RGCs after optic nerve injury, and

explored the key neurotrophic factor in this neuron-protective effect.

MATERIALS AND METHODS

Animals C56BL/6J mice (4-8 weeks old) weighing 30-40 g was obtained from Experimental Animal Center of Kunming Medical University, Yunnan, China. National Institutes of Health (NIH) guidelines for laboratory animal care and safety have been followed. The animals were raised in room-temperature with a 12-hour light/12-hour dark cycle with standard mice chow and clean drinking water. Mice with media opacities, poor blood supply in retina or lack of light reflex were excluded from the study. C56BL/6J mice were treated with intravitreal injection of PBS, BMSCs, BDNF-interference BMSCs (BIM), and GDNF-interference BMSCs (GIM) following optic nerve crush, respectively, with 15 mice in each group.

Optic Nerve Crush Surgical Procedures The surgery process was performed as previously described[32]. Mice were anesthetized with intraperitoneal injection 3.6% chloral hydrate and drops of oxybuprocaine hydrochloride (Benoxil, Santen, Japan). Temporal conjunctival incision was performed. With the separation of extraocular muscles, the optic nerve was fully exposed and clamped at 2-mm to the eyeball for 12s using cross forceps (DUMONT, #N7, Swiss). Erythromycin ointment (Baiyunshan, China) was used postoperatively.

Bone Mesenchymal Stem Cells Isolation and Culture C56BL/6J mice were sacrificed to obtain BMSCs from femurs and tibias. The bone marrow tissue was flushed out by dulbecco's modified eagle medium (DMEM, Gibco, Australia) using 1 mL syringe. After centrifugation (1000 r/min, 10min), the supernatant was discarded. The cells were suspended in DMEM containing 1% penicillin/streptomycin (P/S) and 10% fetal bovine serum (FBS). Cells were then seeded into T25 flasks in a total volume of 5 mL at 37℃ in 5% CO_2. The medium was half changed 72h after seeding. The BMSCs were isolated and purified by adherent screening of cells. CD44 and CD90[33-34] were used to identify BMSCs. The cell morphology and growth was observed by fluorescence microscopy. BMSCs from C56BL/6J mice were used for further experiments after 3-4 passages.

Gene Interference of Bone Mesenchymal Stem Cells SiRNA-BDNF or siRNA-GDNF kit (Ribo biology) was used to silence the expression of BDNF and GDNF in BMSCs. The sequence of siRNA-BDNF was 5'G GUCACAGUCC UAGAGAAAdTdT3'-3'dTdT CCAGUGUC AGGAUCUC UUU5', and the target sequence was GGTCAC AGTCCT AGAGAAA. Moreover, the sequence of siRNA-GDNF was 5'GGGACUCUAAGAUGAAGUUdTdT3'-3'dTdTCCCUGA GAUUCUACUUCAA5', and the target sequence was CCCACT CTAAGATGAAGTT. Fluorescence probe was used to label positive-transfected cells. Cells were divided into 5 groups: 1) normal control cells; 2) negative control group; 3) fluorescence probe group; 4) siRNA-BDNF group (BIM); 5) siRNA-GDNF group (GIM). Transfection buffer 1× (Ribo biology) 300 μL, reagents 9 μL and target siRNA or fluorescence probe 15 μL were mixed separately at room temperature for 15-30min. Then added into cultured cell 100 μL per well andincubated (37℃ 5% CO_2) for 6h before the total medium was changed. Fluorescent images were taken in fluorescence probe group at 6h, 3d and 5d after transfection, to observe the efficiency of transfection. After another 24h, the BMSCs were collected for quantitative polymerase chain reaction (q-PCR) or transplanted into vitreous. The β-actin was used as internal reference.

Intravitreal Transplantation Procedures After optic nerve crush, BMSCs suspensions (BIM or GIM; diluted by PBS) or PBS 2 μL were injected into the vitreous cavity using a micro syringe (Hamilton, 33G, Swiss) 2-3 mm posterior to the limbus temporally. After injection, the needle stayed still for 3-5min and the injection site was immediately covered by conjunctival flap. Erythromycin ointment was locally used to prevent infection.

Tissue Preparation The eyes were removed for different experiments as followings: 1) fixed in 4% paraformaldehyde (PFA) for 12h at 4℃, gradually dehydrated in 15% and 30% sucrose solution for 12h, then used for frozen sections (8 μm, -25℃) and immunofluorescence staining; 2) fixed in 4% PFA for 2h at 4℃, retinas were isolated and used for whole-mount retinas and RGCs counting; 3) fresh retinas were isolated and stored in RIPA lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl，1% NP-40，0.5% sodium deoxycholate, from Thermo Scientific) at -80℃, for the ELISA.

Frozen Sections and Immunofluorescence Eyes were embedded in optimum cutting temperature compound (OCT, Leica) and sectioned at -25 ℃ with a thickness of 8 μm and mo-unted on slides. Optic nerve head was visible in each section. Three sections were randomly chosen for analysis in each eye. The mounted eye sections were dried at 37 ℃ for 3-4h and rinsed in 0.01 mol/L PBS for 3×10min, blocked with 0.3% Triton X-100 in 5% bovine serum albumin for 30min at 37℃. Then the sections were incubated with primary antibody (NeuN, diluted in 2% bovine serum albumin 1:50, ZSGB-BIO, China) for 18-24h at 4℃. The slides were then washed in 0.01 mol/L PBS for 3×10min and incubated with secondary antibody (goat anti mouse, diluted in 0.01 mol/L PBS) at 37℃ for 1-2h. Stained with 4',6-diamidino-2-phenylindole (DAPI), slides were imaged by fluorescence microscope.

Whole-mount Retinas and Immunofluorescence Retinas were cut open in four directions before spread on glass slides. Then they were washed with 0.01 mol/L PBS for 3×10min and blocked with 0.5% Triton X-100 in 5% bovine serum albumin for 4h at 4℃. Then incubated with primary antibody (NeuN, diluted in 2% bovine serum albumin 1:50) for 22-24h at 4℃,

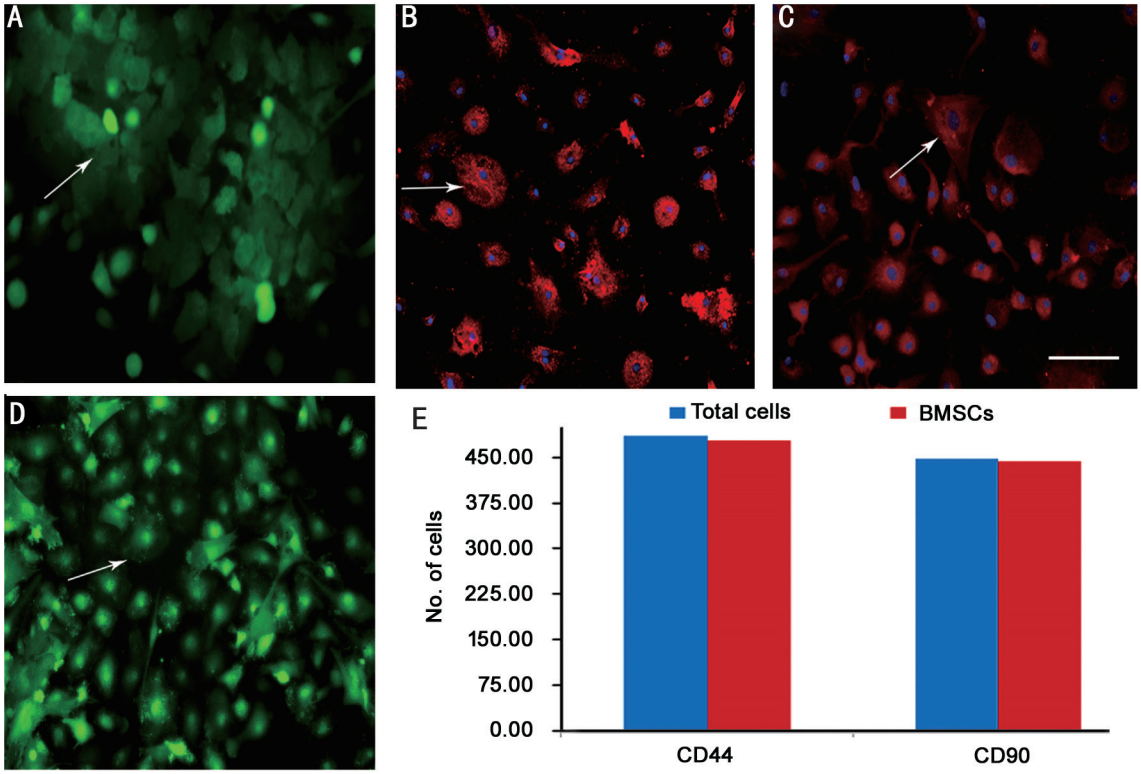

Figure 1 Morphology and identification of BMSCs A: BMSCs after seeding, showed clusters of cells (green); B: BMSCs after 3 passages, appear as poached egg-like, with concentrated distribution (green); C: Identification of CD44+ (red) BMSCs; D: Identification of CD90+ (red) BMSCs; E: Number of total cells and identified BMSCs. Bar: 100 μm shown in picture D.

secondary antibody (goat anti mouse, diluted in 0.01 mol/L PBS) for 2h at 37℃ and imaged by fluorescence microscope.

Microscopy and Analysis The stained sections and whole-mount retinas were analyzed by fluorescent microscope (Leica, Germany). RGCs were recognized as positive in both NeuN and DAPI and distributed in the first monolayer of cells in retina. The morphology of RGCs were recorded by the frozen sections and measured in images taken at 400× magnification. The quantification of surviving RGCs was similar to previous studies[35-36] and made directly from the images of whole-mount retinas with 200× magnification, with 6 fields in each quadrant, and the results were shown as RGCs/mm^2.

ELISA of BDNF Protein in Retinas After ultrasonic cell disruption and centrifugation (12 000 r/min, 10min, at 4℃), 2 μL of the supernatant was collected to test the concentration of proteins by micro-plate reader (Bio-Tek). The remaining supernatant was used to test the BDNF protein level by ELISA, following the instructions of BDNF ELISA Kit of R&D.

Statistical Analysis Statistical analysis was performed with SPSS 22.0 software and the data was shown as mean±SD. The results were analyzed by one-way ANOVA and the comparison was performed by the LSD method. P<0.05 was considered statistically significant.

RESULTS

Identification of Bone Mesenchymal Stem Cells The morphology of BMSCs was as shown in Figure 1. CD44 and CD90 were both used to mark BMSCs. As a result, about 98% cells after 3 passages exhibited both positive staining, shown in Figure 1.

Efficiency of siRNA in Bone Mesenchymal Stem Cells Detected by fluorescent probe, the efficiency of transfection by reagent was over 99% at 6h, 3d and 5d after transfection (Figure 2).

After siRNA transfection, q-PCR of BDNF and GDNF in BMSCs showed that the relative expressions 24h post transfection were $(0.674\pm0.035)\times10^{-4}$ and $(6.926\pm0.649)\times10^{-4}$. Meanwhile, the relative expressions of BDNF and GDNF in normal BMSCs were $(4.079\pm0.151)\times10^{-4}$ and $(12.138\pm0.339)\times10^{-4}$. Expression of BDNF and GDEF was effectively reduced by siRNA at 24h so much as 72h (P=0.000; Figure 3).

Neuro-protective Effects of Bone Mesenchymal Stem Cells In mice retinas, RGCs appeared monolayer with consistent arrangement. ONC and PBS injection both caused obvious discontinuities in RGCs layer, while BMSCs transplantation improved the consistence of survived RGCs (Figure 4). As the whole-mount retinas showed in Figure 5, the average density of RGCs significantly decreased to 410.77±56.70/mm^2 21d after ONC operation, compared to 1351.39±195.97/mm^2 in control group (P<0.05). Intravitreal transplantation of BMSCs significantly increased RGCs with a density of 625.07±89.64/mm^2 (P<0.05). But RGCs with BIM treatment was 354.07±39.77/mm^2, and GIM treatment was 326.67+33.37/mm^2 (P=0.024). Both were significantly reduced (P<0.05). Density of RGCs in ONC group was even higher than that of BIM or GIM treatment (P<0.05).

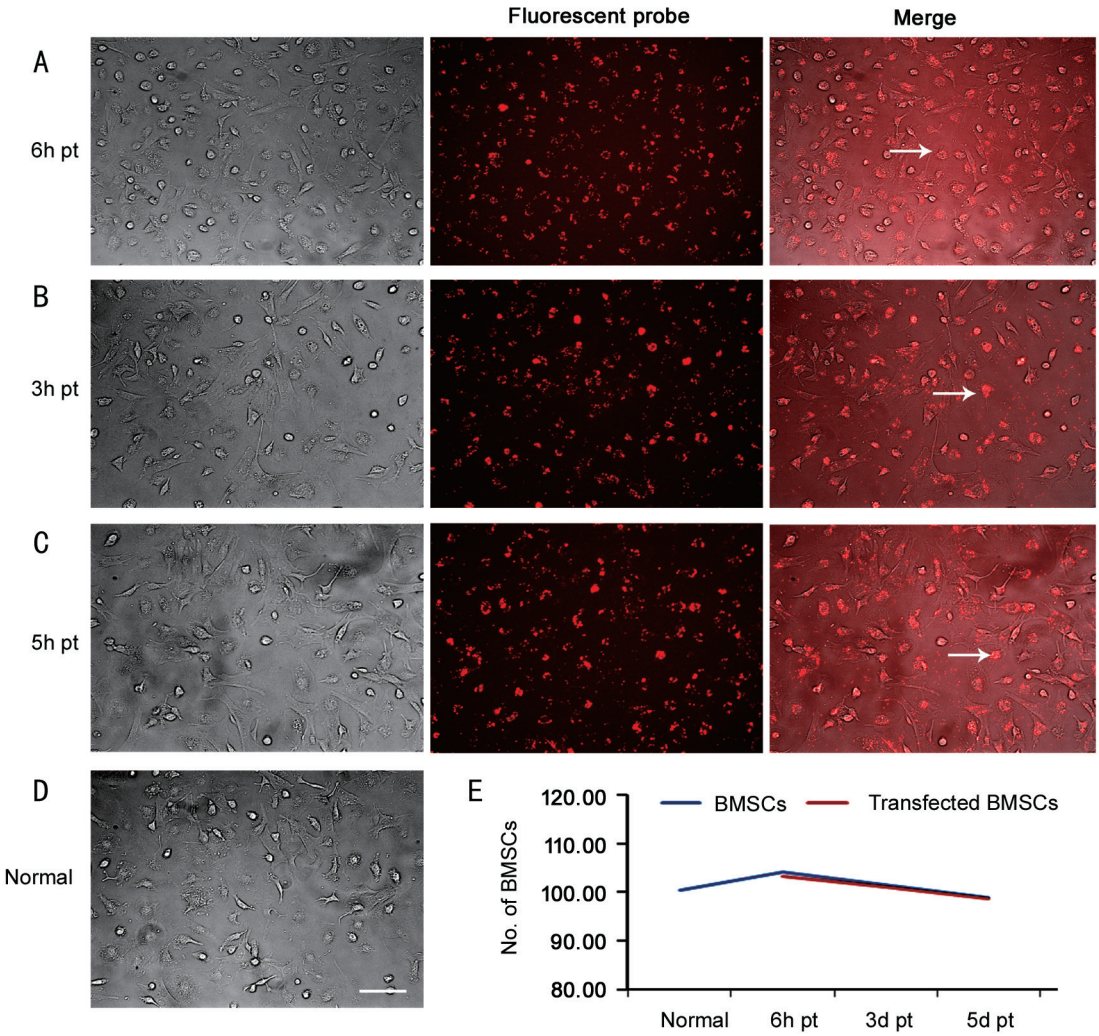

Figure 2 BMSCs before and after transfection A-D: Morphology of BMSCs at 6h, 3 and 5d after transfection and before transfection; E: Number of total and transfected BMSCs in each group per field (×200). Bar: 100 μm shown in picture D. pt: Post-transfection. Arrow: Transfected BMSCs with positive fluorescence (red).

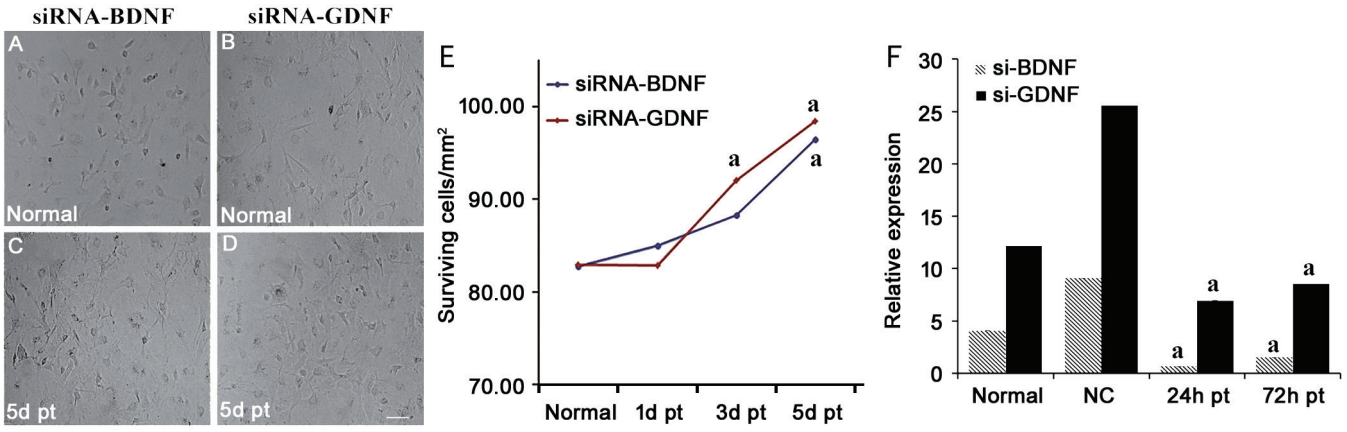

Figure 3 BDNF and GDNF expression in BMSCs before and after siRNA transfection A, B: BMSCs before transfection; C, D: There was no obvious cell death 5d after transfection; E: Surviving BMSCs per field before and after transfection at each time point; F: Relative expressions ($\times 10^{-4}$) of BDNF or GDNF in each group. $^{a}P<0.05$ *vs* normal group. pt: Post transfection. NC: Negative control. Bar: 100 μm shown in picture D.

Brain-derived Neurotrophic Factor Protein Level in Retina In mice retinas, the BDNF was 25.25±2.07 ng/L and reduced to 22.71±1.48 ng/L 21d after optic nerve crush, with no significant difference (P=0.170). PBS caused lower expression of BDNF (14.86±1.13 ng/L) in retinas. BMSCs transplantation significantly increased BDNF in retinas (81.27±3.18 ng/L) by almost 3 times compared to control or ONC group (P=0.000). However, retina BDNF was not increased by BIM or GIM, which was 29.63±2.15 ng/L and 25.72±2.07 ng/L, respectively (Figure 6).

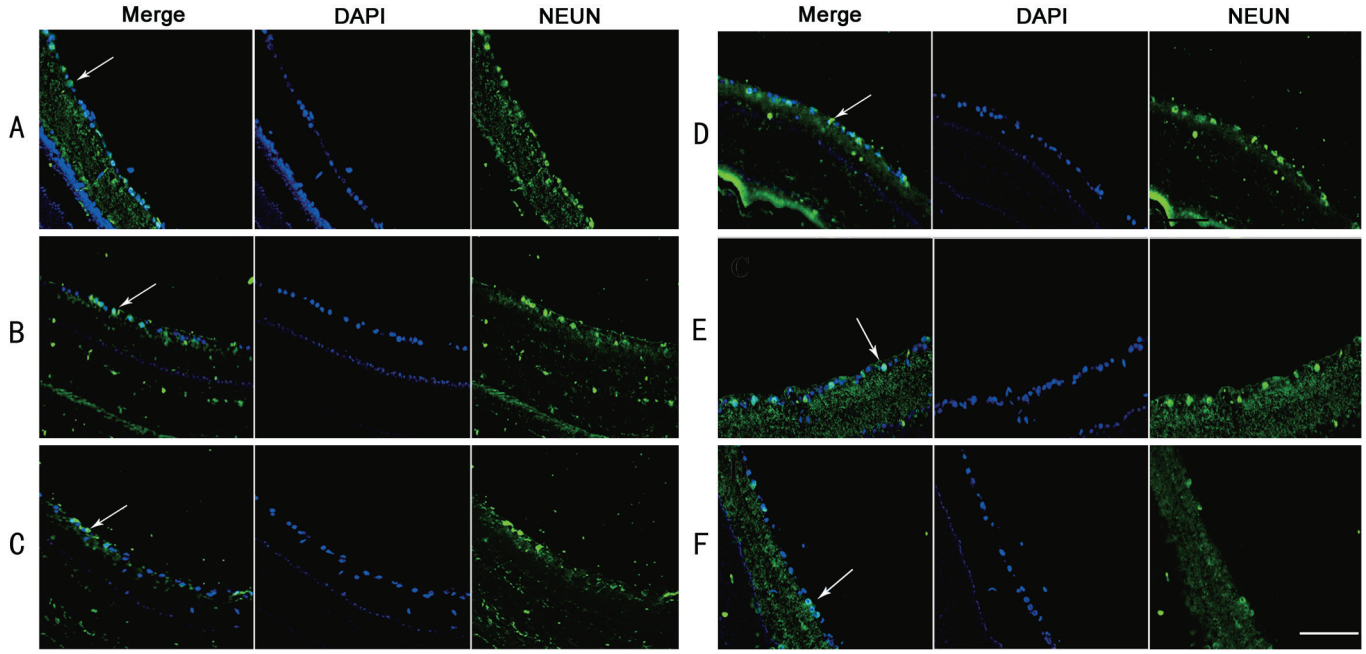

Figure 4 Frozen sections of morphological changes of RGCs A: Monolayer and consistent arrangement of RGCs in control normal retina; B: Three weeks after ONC, there was a loss of RGCs in the retina, no consistent arrangement; C: After ONC and PBS injection, the morphology was similar to B; D: After BMSCs transplantation, the consistence in arrangement of RGCs was improved; E: BIM treatment decreased the consistence of NEUN+ RGCs; F: GIM treatment also decreased the consistence of RGCs even significant than BIM. ONC: Optic nerve crush; BIM and GIM: siRNA-BDNF or siRNA-GDNF transfected BMSCs. Arrow: Surviving RGCs with both DAPI and NEUN positive. Bar: 100 μm shown in last picture.

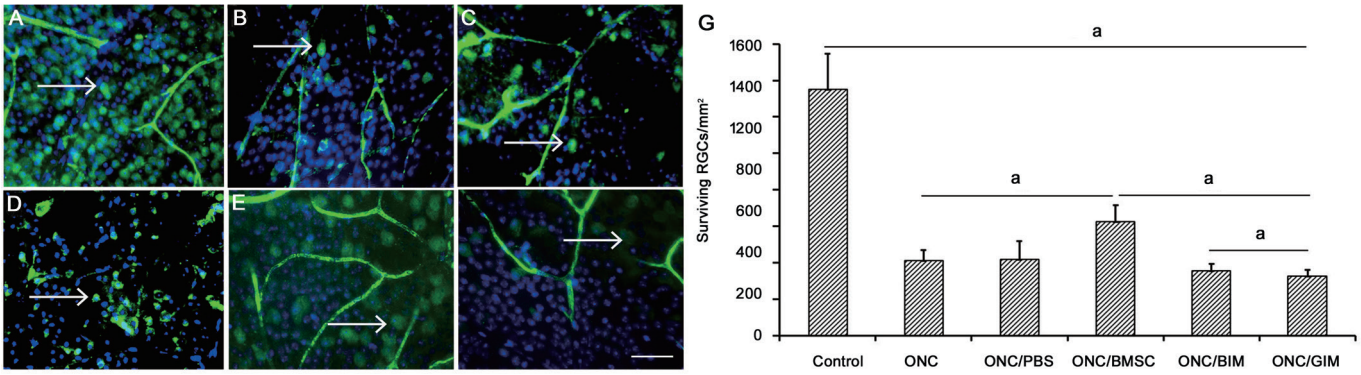

Figure 5 Whole-mount retinas of surviving RGCs A: Well-distributed RGCs in control normal retina; B: RGCs were significantly reduced and distributed unevenly 3wk after ONC; C: After ONC and PBS injection, distribution of surviving RGCs was similar to B; D: After BMSCs transplantation, RGCs were more densed and better distributed than B and C; E: BIM treatment decrease the density of RGCs; F: GIM treatment also decrease the number of RGCs even significant than BIM; G: The number of surviving cells in different groups. Con: Control; ONC: Optic nerve crush. BIM or GIM: siRNA-BDNF or siRNA-GDNF transfected BMSCs. Arrow: Surviving RGCs with both DAPI and NEUN positive. [a]P<0.05. Bar: 100 μm shown in last picture.

DISCUSSION

In previous studies, the methods for marking RGCs include retrograde and immune markers. The most commonly used retrograde marker was fluorescence gold[35], which principle was that RGCs axons can in-take the fluorescence gold injected into the superior colliculus and transport to cell body. This method was highly specific. The most common antibodies for immune markers were Tuj1 (βIII tubulin), Brn3[37], NeuN and Thy-1. Immunostaining was a much simpler method, but its specificity and stability might be less than retrograde labeling. Due to the complexity of the retrograde labeling which easily leads to animal death, our study used a relatively simpler method of immunofluorescence. In selection of antibodies, NeuN was an antibody that could specifically recognize neurons, which selectively labeled nucleus and pericaryon[38] and was commonly used to stain neurons in neurodegenerative diseases[39]. Researchers also found that[40], when cortical neurons were stained, NeuN was better than Nissl staining in neuronal density, size and shape. Tuj1 was also commonly used for marking RGCs, mainly marked cytoskeletal that

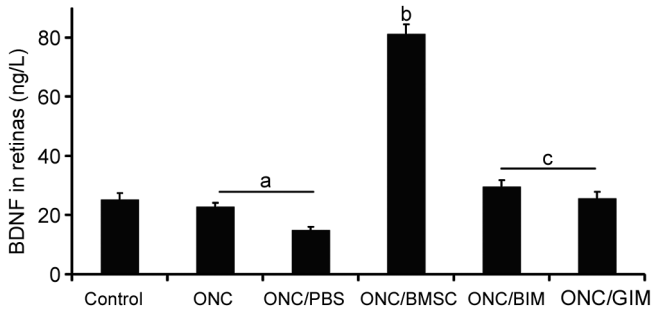

Figure 6 BDNF protein in retinas by ELISA [a]*P*=0.000; [b]*P*=0.000 *vs* other groups; [c]*P*=0.04.

could clearly show the details of axons. In our preliminary experiments, we found that although Tuj1 was a good marker for RGCs in frozen sections, but in whole-mount retinas, staining of cell bodies and axons were complexly interweaved and hard to quantify RGCs. NeuN was widely used to label RGCs in recent years[41-43]. By co-localization of NeuN and βIII Tubulin, Osborne *et al*[44] found that both transverse section and whole-mount staining confirmed a strong level of co-localization. Their study confirmed that NeuN, βIII Tubulin and Thy-1 were all appropriate for labeling RGCs in retinas. In another study, strong co-labelling was also detected between NeuN and fluorogold retrograde labeled RGCs[45]. It was important to notice that NeuN did not label amacrine cells as there was no major staining of NeuN in the inner nuclear layer[46]. Therefore, NeuN antibody was chosen to label RGCs, which clearly stained RGCs cell layer, both in the frozen sections and whole-mount retinas.

BMSCs are important stem cells and pluripotent to differentiate into bone, cartilage, muscle cells, endothelial cells and nerve cells[47]. They are used to treat diseases of nervous systems in recent years.

In our experiment, transplantation of BMSCs exhibited an optimal effect on the RGCs survival, showed by cell numbers and consistence in morphology. Moreover, BIM and GIM can inhibit their protective effect. Our experiment indicated that BDNF and GDNF as critical neurotrophic factors in BMSCs played important protective roles to the retina. In 2004, researchers[48] found that GDNF expression was low in BMSCs, but it was essential for repair of nervous system. In 2007, Jiang *et al*[12] established hypertension glaucoma model and injected GDNF microsphere into vitreous body, and found that persistent secretion of GDNF could effectively protect RGCs. *In vitro* studies, Taylor *et al*[49] cultured retinas with GDNF and found that GDNF can reduce the degeneration of cells. BDNF was also found to be effective in RGCs survival after optic nerve trauma in cat retinas[50], and could further reserve the morphology of cell body and axons[51]. However, the number of axons in BDNF knockout mice and normal mice were almost the same, this suggested exogenous BDNF supplements could reduce the degeneration of RGCs, but endogenous BDNF was not essential for RGCs survival[52].

Zhao *et al*[53] found that after ONC in rats, cord blood stem cell transplantation can increase BDNF mRNA expression of retina. This was similar to that of our results that BMSCs transplantation significantly improved the BDNF expression in retinas. These results indicated that stem cell transplantation might stimulate retinal BDNF expression through some signal pathways, so as to protect neurons. Deng *et al*[54] established a rat model of cerebral hemorrhage with BMSCs or GDNF modified BMSCs were transplanted in injury site. They found that BMSCs and GDNF modified BMSCs both up-regulated the mRNA of BDNF, BDNF receptor (TrkB) and BDNF protein, especially the modified BMSCs. In 2011, Peng *et al*'s study[55] found that, GDNF could increase BDNF expressing by 1.4 times in neurons *in vitro*. These findings suggested that GDNF might be the key factor in BMSCs, likely *via* up-regulating endogenous BDNF expression.

The limitation of our research was absent BDNF knock-out mice to further prove the effect of endogenous BDNF in protecting RGCs. In the future study, more research was needed to investigate the certain pathway of BDNF and GDNF in BMSCs and explore its neuro-protective effect on RGCs.

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