Gender difference on neuroprotective effect of rat BMSCs against the hypoxia-induced apoptosis of RGCs

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Abstract

Purpose: Bone marrow mesenchymal stem cells (BMSCs) have been shown neuro-protective effects in several disease models. However, no studies have examined whether gender influences the neuro-protective effects of BMSCs. Here, we explore the different neuro-protective effects of BMSCs due to gender source against hypoxia-induced apoptosis of retinal ganglion cells (RGCs).

Methods: BMSCs was cultured from female and male juvenile rats by gradient density centrifugation and identified. The retinal tissues of newborn rats were prepared with enzymatic digestion to acquire primary RGCs. The RGCs were then co-cultured across a membrane insert with BMSCs in the transwell system under hypoxia injury. Cell apoptosis was detected using AnnexinV-FITC/PI assays and caspase-3 activity assay.

Results: Our experiments demonstrated that there were marked increases in the percentage of apoptotic RGCs and the caspase-3 activity after 24 h under hypoxia condition compared with that under the normoxia condition ($P < 0.05$). When co-cultured with both female BMSCs and male BMSCs under hypoxic condition, the percentage of apoptotic RGCs and the caspase-3 activity had significant decreases compared with that when culture alone (both $P < 0.05$), and had a significant difference between the female BMSCs and male BMSCs ($P < 0.05$).

Conclusion: Our results indicated that BMSCs have neuro-protective effects against hypoxia-induced apoptosis of RGCs, and female BMSCs have a greater neuro-protective ability compared with male BMSCs. A further understanding of the cellular biology underlying these differences may contribute to the development of new therapeutic strategies for neurological repair and regeneration.

Key Words: Bone marrow mesenchymal stem cell; retinal ganglion cells; neuro-protection; hypoxia injury; gender difference

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MATERIALS AND METHODS

Design
A comparative observation at the cellular and molecular levels.

Time and setting
The experiments were performed at the Eye Laboratory of Renmin Hospital, Wuhan University, China from Jun 2013 to Jun 2014.

Materials
Healthy Sprague-Dawley rats were obtained from the laboratory Animal Center of Renmin Hospital of Wuhan University, Wuhan, China. The rats used in all experiments were about 3-6 months of age and equivalent in weight or newborn. All the animal experiments were conducted in accordance with the national guidelines for the care and use of laboratory animals. This study was approved by the Ethnic Committee of Renmin Hospital of Wuhan University.

Methods

Isolation and culture of rat BMSCs
Bone marrow cells were obtained from twenty healthy female and male rats and characterized as previously described [48]. Briefly, bone marrow aspirate were collected from rat’s femurs and tibias. Bone marrow was flushed out using Dulbecco’s modified Eagle’s medium with low glucose (L-DMEM) (Gibco, USA). Suspended cells were centrifuged at 1000 rpm for 5 min. After discarding the supernatant, cells was resuspended in L-DMEM with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.4 mg/ml HEPES, and 3.7 mg/ml NaHCO3. Then, they were plated in a 25 cm2 culture flask and incubated at 37 °C in 5% CO2. After 12 h, the non-adherent cells were moved. The culture medium was changed every two days. On Day 12 or 13, the confluent cultures (Passage 0; P0) were trypsinized with 0.25% trypsin in 0.02% ethylenediaminetetraacetic acid (EDTA) and subcultured as P1. The acquired BMSCs were confirmed when they differentiated into osteocytes and adipocytes after the additional adding of specific differentiation media as before [46], and the immunophenotype was assayed by flow cytometry after a co-incubation with fluorescein isothiocyanate (FITC)/phycoerythrin (PE)-conjugated monoclonal antibodies including CD29, CD34, CD44, CD45, CD80 and CD86 (BD Biosciences, Sparks, MD, USA) as described previously [49]. In subsequent experiments, cells at P3-6 were used for the neuroprotection assays.

Purification and Culture of RGCs
Primary retinal ganglion cells (RGCs) were purified and cultured as described previously [50]. Briefly, newborn rats were sacrificed and retinae were dissected and incubated for 45 min in Dulbecco’s phosphate buffered saline (D-PBS) supplemented with 160U/ml papain and 200 U/ml DNase. The retinal tissue was then sequentially triturated in D-PBS containing 0.2% bovine serum albumin (BSA, Gibco) and 650 U/ml DNase. Cells were pelleted and resuspended in D-PBS/0.2% BSA and then purified by a two-step immunopanning procedure. That is, the dissociated retinal cells were incubated in plates coated with an anti-rat macrophage monoclonal antibody (1:50) to exclude macrophages, and were then incubated in plates coated with an anti-rat Thy1 monoclonal antibody (1:300). RGCs that adhered to the plates were collected by centrifugation at 600 rpm for 5 min and were seeded on 13 mm glass coverslips in a 24-well plate that had been coated with 50 μg/ml poly-L-lysine (Sigma, St. Louis, MO) and 1 μg/ml laminin (Invitrogen, Carlsbad, CA). Purified RGCs were plated at a density of approximately 1,000 cells per well and cultured in neruobasal medium (Invitrogen) supplemented by 100μg/ml BSA, 1 mM L-glutamine (Sigma), B27 (Invitrogen), 100U/ml penicillin, 100 μg/ml streptomycin, 10 μM forskolin (Sigma), 40 ng/ml human recombinant BDNF and 40 ng/ml rat recombinant CNTF (Sigma) with a humidified atmosphere containing 5% CO2 and 95% air at 37°C for 7 days. Cells were monitored with Zeiss HAL100 microscope (Carl Zeiss Microscopy, Jena, Germany) for the morphological changes during growth. The acquired retinal cells were confirmed to be retinal ganglion cells by immunocytochemical staining of Thy1. The coverslips carrying viable RGCs were only used in case of distinct neurite sprouting, which was usually after 7 days of incubation. Only cultures of pure RGCs with no trace of glial contamination were selected for the study.

RGCs cell co-culture with BMSCs and Hypoxic injury
BMSCs (P3-6) were co-culture with RGCs. BMSCs were separated from RGCs with 0.4 μm porous polystyrene membrane in 12 well plates with 12 mm trans-wells. This co-culture system allowed the cells to maintain crosstalk mediated by secretion of signal molecules, meanwhile it avoided mixture of two cell types and physical contact in during culture time. RGCs were cultured in 12 well plates for 7 days in vitro prior to hypoxic injury. BMSCs were plated in 12 well inserts and grown to 80–100 % confluence in serum-containing growth medium. Prior to placing BMSCs-containing inserts into plates containing RGCs, BMSCS were rinsed twice with PBS and medium was changed to neuorobasal medium, consequently removing serum from the co-culture. For hypoxia stress [51], the co-culture system were transferred to a controlled-atmosphere incubator containing 5% CO2, 90% N2, and 5% O2 mixture and co-cultured for 24 h. In the experiments, RGCs in matching controls were incubated at 37°C incubator with 95% air and 5% CO2. Then, RGCs apoptosis were assessed using Flow cytometry analysis and caspase-3 activity assay.

Immunocytochemistry
RGCs were plated on coverslips and were washed twice with phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde for 20 minutes at room temperature. The cells were soaked in blocking buffer containing 0.5% Triton X-100, 5% bovine serum in phosphate buffer for 25 minutes at room temperature. Then cells were incubated with primary anti-
bodies at 4°C overnight, washed, incubated for 2 hours at 37°C with secondary antibodies conjugated to a fluorescence marker, rinsed, mounted, and then observed under a confocal laser microscope (Leica, TCS, SP5, Germany). Primary antibodies included anti-Thy1 (1:300; Abcam Inc., Cambridge, MA, USA).

**Flow cytometry analysis for cell apoptosis**

The percentages of apoptotic or necrotic cells following hypoxia were determined by flow cytometry using Annexin V-FITC/Propidium iodide (PI) apoptosis detection kit (Becton Dickinson, Rahway, NJ, USA) according to the manufacturer’s instructions. Briefly, cells (1 × 105 cells/mL) attached to the bottom of plates and the cells in the supernatant were collected, washed twice with ice-cold PBS and re-suspended in 200 μL of Annexin binding buffer. Then, 2 μL of Annexin V-FITC and 2 μL of PI were added and incubated for 5–15 minutes in the dark at room temperature. After incubation, the samples were analyzed by flow cytometry (FACS Aria™, BD, San Jose, CA, USA). Viable cells are negative for both PI and annexin V; early stage apoptotic cells are positive for annexin V and negative for PI, late stage apoptotic cells are positive for both annexin V and PI, and both are regarded as apoptotic cells.

**Caspase-3 activity analysis for cell apoptosis**

Caspase-3 activity was determined by using the CaspACETM colorimetric assay system (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, cells (1 × 106 cells/mL) attached to the bottom of plates and the cells in the supernatant were collected, washed twice PBS and re-suspended in 400 μL of caspase assay buffer and 2 μL of 10 mM DEVD-pNA substrate and incubated for 4 hours at 37°C. After incubation, absorbance of the samples were analyzed by subtraction of the mean absorbance of the blank from that of the sample using a microplate reader at 405 nm.

**Statistical analysis**

All experiments were performed at least three times. Results of representative experiments are presented. Descriptive statistics included mean ± SD. Comparisons between two groups were performed using t-tests. A p value <0.05 was considered statistically significant.

**RESULTS**

**Culture and identification rat BMSCs**

A mesenchymal stem cell population originating from adult rat bone marrow from female and male donors were successfully isolated, purified and expanded in monolayer culture. After reaching confluence and subsequent sub-cultivation at early passage, Female BMSCs (FBSMCs) and male BMSCs (MBMSCs) became morphologically homogeneous fibroblast-like cells (Figure 1A, B). After long-term culture (≥10 passages), BMSCs began to exhibit morphological changes mixed with bipolar or dendrite-like shapes, and then died gradually as the culture time extended. Interesting, we observed that FBMSCs were able to maintain subculture with more passage compared to male BMSCs (Figure C, D). Flow cytometric analysis demonstrated the expression of CD29 and CD44 but not CD34, CD45, CD80 and CD86 (data not shown). In addition, FBMSCs and MBMSCs underwent appropriate differentiation into adipocytes and osteoblasts after culture in induction media (Figure 1E–H).

**Caspase Activity Assay**

Caspase-3 activity assay was further used to evaluate the neuroprotection of BMSCs and compare the gender difference of their anti-apoptotic effects in hypoxia-induced apoptosis of RGCs by calculating the percentages of apoptotic or necrotic cells (Figure 3). Apoptotic cells included early apoptotic cells (Annexin V+/PI−) and late apoptotic cells (Annexin V+/PI+). Our experiments demonstrated that there were marked increases in the percentage of apoptotic cells after 24 h under hypoxia condition compared with that under the normoxia condition (51.85±4.8% vs. 8.64±1.22%, P = 0.000). When co-cultured with both FBMSCs and MBMSCs under hypoxic condition, the percentage of apoptotic RGCs detected significantly decreased compared with that when culture alone (25.15±3.5% vs. 51.85±4.8%, P = 0.000 and 37.94±2.98% vs. 51.85±4.8%, P = 0.001, respectively), and the percentage of apoptotic cells had a significant difference between the FBMSCs and MBMSCs (P = 0.002, n = 3). The results showed that BMSCs effectively inhibited hypoxia-induced apoptotic of RGCs and the neuroprotection effects presented the gender difference.

**BMSCs protects RGCs from hypoxia induced apoptosis and the gender difference**

**Flow Cytometry Assay**

Flow Cytometry assay was used to evaluate the neuroprotection of BMSCs and compare the gender difference of their anti-apoptotic effects in hypoxia-induced apoptosis of RGCs by calculating the percentages of apoptotic or necrotic cells (Figure 3). Apoptotic cells included early apoptotic cells (Annexin V+/PI−) and late apoptotic cells (Annexin V+/PI+). Our experiments demonstrated that there were marked increases in the percentage of apoptotic cells after 24 h under hypoxia condition compared with that under the normoxia condition (51.85±4.8% vs. 8.64±1.22%, P = 0.000). When co-cultured with both FBMSCs and MBMSCs under hypoxic condition, the percentage of apoptotic RGCs detected significantly decreased compared with that when culture alone (25.15±3.5% vs. 51.85±4.8%, P = 0.000 and 37.94±2.98% vs. 51.85±4.8%, P = 0.001, respectively), and the percentage of apoptotic cells had a significant difference between the FBMSCs and MBMSCs (P = 0.002, n = 3). The results showed that BMSCs effectively inhibited hypoxia-induced apoptotic of RGCs and the neuroprotection effects presented the gender difference.
the normoxia condition (0.209±0.023 vs. 0.012±0.008 $P = 0.01$). When co-cultured with both FBMSCs and MBMSCs under hypoxic condition, the caspase-3 activity had significant decreases compared with that when culture alone (0.100±0.006, 0.122±0.011, respectively, both $P = 0.01$), and the caspase-3 activity had a significant difference between the FBMSCs and MBMSCs ($P = 0.01$, $n = 3$). The results showed that BMSCs effectively inhibited hypoxia-induced apoptotic of RGCs and the neuro-protection effects presented gender difference.

**Discussion**

The death of retinal ganglion cells is the final common pathway of vision loss in many blinding optic neurodegenerative disease such as glaucoma, age-related macular degeneration (AMD), diabetic retinopathy, optic neuritis, ischemic optic neuropathy and various inherited retinal dystrophies. These diseases can occur when RGCs are exposed to hypoxic conditions due to RGCs are particularly sensitive to acute, transient, and mild systemic hypoxic stress [13]. As a common cause for neurotoxicity in a variety of acute and chronic neurodegenerative diseases [13], hypoxia can induces the expression of vascular endothelial growth factor (VEGF) [13-15], nitric oxide (NO) [13,16], excitatory amino acid (glutamate) [13,17], inflammatory cytokines and reactive oxygen species (ROS) [18]. These processes result in the apoptosis or necrosis of RGCs through various mechanisms such as disruption of blood-retinal barrier (BRB), excitotoxicity and increased accumulation of intracellular Ca2+ [13]. In addition, free-radicals generated in hypoxic-ischemic conditions result in RGC loss because of an imbalance between antioxidant- and oxidant-generating systems [19]. Therefore, it is important to reduce the death of retinal ganglion cells and develop neuro-protective strategies for retinal ganglion cells against hypoxia injury.

The use of stem cells in neuro-protective treatments in all areas of medicine has been under recent consideration. The retinal neuro-protective properties of MSCs have been demonstrated in various studies [20]. Mesenchymal stem cells secrete brain derived neurotrophic factor (BDNF), nerve growth factor, glial cell-derived neurotrophic factor (GDNF), cilary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF) and various cytokines and neurotrophic factors [21-24]. These factors secreted from MSCs have been shown to provide powerful neuro-protective ef-
Figure 3 The effect of co-culture with MBMSCs and FBMSCs on the hypoxia-induced apoptosis of RGCs.

A: Annexin V-FITC/PI staining using flow cytometry. A control normoxic culture is shown in (a). Cells were exposed to hypoxia (b) for 24 hours either alone or in the presence co-culture with MBMSCs (c) or FBMSCs (d). Annexin V+/PI− (Q3) demonstrates early apoptotic cells, and Annexin V+/PI+ (Q2) demonstrates late apoptotic cells. B: Quantitative analysis of the percentage of apoptotic cells. Apoptotic cells included early apoptotic cells (Q3) and late apoptotic cells (Q2). The data are shown as the means ± SD of three independent experiments.* P<0.05 as compared to the normoxia group, ※ P<0.05 as compared to the hypoxic group, ★ P<0.05 as compared to the hypoxic + MBMSCs.

Effects on models of Central nervous system (CNS) disorders, such as amyotrophic lateral sclerosis [25], multiple sclerosis [25-29], Parkinson disease [30], ischemia [31,32], spinal cord injury [33,34]. The studies involving the use of MSCs in glaucoma treatment showed that MSCs were effective in sustaining RGCs viability in animal models [35-37]. The first clinical trial using bone marrow-derived MSCs on glaucoma (Stem Cell Ophthalmology Treatment Study (SCOTS)) has been ongoing from August 2013 (NCT01920867; http://clinicaltrials.gov/; Table 2) [20]. However, the gender difference on the neuro-protection effects of BMSCs has not been investigated. Therefore, a better knowledge of the sex-specific difference in neuro-protection effects mediated by BMSCs is a prerequisite before considering their use in the treatment of neurological diseases. Here, we investigated the neuro-protection effects of BMSCs and compared the effects from each sex. These results provided crucial information for future application of BMSCs for successful RGCs neuroprotection.

First, we isolated rat BMSCs from juvenile female and male rats, thereby establishing a common experimental animal model to study the gender differences of BMSCs. Through observation of the cellular morphology, we observed that their shape showed a uniform fibroblast-like shape after subculture at early passages. With the increase of passage number, the cells gradually lost their vitality, but the FBMSCs maintained longer passage number compared with MBMSCs showing that female seem to have a stronger viability of subculture. The adherent cells were verified to be mesenchymal stem cells derived from bone marrow due to their ability to undergo lipogenic and osteogenic differentiation and their representative expression of CD34+. The difference with characteristic uniform shape of BMSCs is that it is difficult to obtain uniform shape during culture process of primary RGCs. In the area of high density, cells expanded and connected with long neurites as the culture time extended. But in the area of low density, cells presented round or oval shape with short neurites and died gradually as the culture time extended. It also suggested that RGCs is high vulnerable and sensitive to the disturbance in the ambient environment, which is related to the nature of differentiated neurons. That is the reason that we can easily observe RGCs apoptosis or necrosis when cells were directly exposed to hypoxia in our study, confirming that RGCs are exquisitely sensitive to hypoxia.

Further, Our RGCs-BMSCs co-culture model reflected that BMSCs are able to exert a protective effect on the viability of RGCs against hypoxia injury. Using Flow Cytometry and caspase-3 activity assay, we can significantly observed co-culture with both FBMSCs and MBMSCs suppressed the hypoxia-induced apoptosis of RGCs and improved their survival. This feature is in agreement with previous reports indicating that the rate of hypoxia induced RGCs loss is reduced when the BMSCs were intravitreally transplanted in an experimental oculat hypertension (OHT) model [20,38]. Therefore we conclude that BMSCs protect RGCs from hypoxia-induced apoptosis. The action mechanism of BMSCs in neuroprotection might originate from the secretion of neurotrophic factors, including BDNF, GDNF, CNTF and bFGF, which may play an important paracrine role in modulating the inflammatory processes, regulating axonal re-growth along with neuronal repair and activating endogenous repair mechanisms [39,40].

Moreover, we have focused on the comparison of neuro-protective properties of BMSCs due to gender source. Few studies have addressed whether gender differences can have an influence in stem cell function and repair. In our previous study, we have found that female rhesus monkey BMSCs can acquire higher neurogenic potential compared to male rhesus monkey BMSCs under the differentiation condition [41]. In the present study, we observed that rat female BMSCs exhibited decreased apoptosis of RGCs compared to male...
BMSCs in response to hypoxia, which suggested female BMSCs have greater neuroprotection ability. This is a very interesting observation, which may be attributable to the following reasons: 1. Female BMSCs may demonstrate improved attenuation of hypoxia injury or other insult compared with male BMSCs because of their inherent greater ability to survive [42], which can be explained from our result showing that female BMSCs could maintain stronger viability as the subculture number extended. 2. Female BMSCs may release greater levels of protective factors and decrease more inflammatory cytokines in response to hypoxia or other injury [42]. This study and our previous investigation suggest that gender plays an important role in the stem cell function, including their neurogenic potential and neuroprotection properties. The gender difference may be associated with the role of estrogen. Estrogen can enhance female stem cell survival and activity [43]. The effect may be mediated by mitogen activated protein kinases and cyclin dependent kinases [44]. Exogenous estrogen, possibly via estrogen receptor alpha, has been shown to increase MSCs function and calcium deposition [45,46]. Further, gender differences in TNF-R1 signaling may also account for the enhanced VEGF and decreased TNF- expression seen in female MSCs. TNF-R1 ablation also significantly reduces male MSC apoptosis after hypoxic injury. Thus sex hormones and various intracellular signaling pathways may in part explain the gender differences in stem cell function described previously and currently. The gender difference, although observed in vitro, could provide information on the neuroprotection ability of BMSCs and their intrinsic commitment in vivo. Based on these experimental evidences, it is possible that this observation reflects an expansion of more active neuronal growth in females compared to males. For example, it may explain the epidemiological observations that women have a lower incidence of symptomatic Parkinson’s disease and a higher age of onset than men due to their higher physiological neuron levels [47], which may acquire greater level neuroprotection partly by bone marrow grafts of progenitors (e.g., BMSCs) having migrated from the blood into the brain parenchyma across the blood–brain barrier. Although the role of host sex was not examined in this study, stem-cell sex differences in neuro-protective properties could lead to different clinical treatments for neurological diseases in women and men. However, some limitations of the present study should be noted, such as the age, low number of experimental animals, chronic injury model, and the exact mechanism underlying this protective effect. In addition, Rats are 80% homologous to human, so the results from human derived cells experiments need to be performed to verify the gender difference correlated to events that occur in humans. Therefore, further studies on the neuro-protective properties and intrinsic mechanisms underlying the observed differences should be performed and accompanied by an age hierarchy of donors, more samples, model design, and the mechanism so as to further our understanding of the cell biology and better the clinical application of these cells.

In summary, our data suggested that both male and female rat BMSCs are able to enhance the survival of RGCs under the hypoxic condition, and female BMSCs confer greater neuroprotection effects compared to male BMSCs after hypoxic injury. These results further the understanding of the neuroprotection effects of BMSCs and may contribute to the development of new therapeutic strategies for neurological repair and regeneration.

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Conflicts of interest: None declared.

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