Int J Clin Exp Pathol 2020;13(6):1397-1402
www.ijcep.com /ISSN:1936-2625/IJCEP0096709

Original Article

Effects of bone marrow mesenchymal stem cells transplantation on organoretinal culture after hypoxia injury

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Received May 8, 2019; Accepted June 20, 2019; Epub June 1, 2020; Published June 15, 2020

Abstract: Objective: To investigate the effects of bone marrow mesenchymal stem cells transplantation on organoretinal cultures after a hypoxia injury. Method: The retinal tissues were cultured in vitro and then transplanted with bone marrow mesenchymal stem cells. Then, H&E staining and immunohistochemical assay were conducted to investigate the changes in retinal tissue structure and the migration and differentiation of stem cells. Results: The retinal tissues were slightly damaged in the stem cell transplantation group; the control group, the retinal tissue structure was changed, and the thinning of their thickness was clearly evident. The transplanted stem cells can migrate to each layer of the retina to replace the damaged dead cells, which can protect the peripheral injured tissues and cells.

Keywords: Retina, organ culture, stem cell transplantation, migration, replacement

Introduction

With the development of a social economy and the improvement of medical technology, blinding diseases, such as cataracts and ocular surface infectious diseases, have been well controlled and clinically treated. However, the incidence of age-related retinopathy [1] and diabetic retinopathy [2] has continued to be a risk with the aging of the population and lifestyle changes. It is a problem worthy of study: how to effectively prevent and interfere with retinal diseases in their early stages. In addition, retinal tissue is an ideal model for the study of the development of the central nervous system (CNS) because of its easy access, clear hierarchical structure, and fixed cell type [3-7]. Therefore, in this study the retinal tissue was cultured in three-dimensional in vitro, which is of great significance for exploring the pathogenesis of retinal diseases, screening effective intervention measures, and studying the development of central nervous system subsystems.

Many diseases are attributed to pathological changes in neurons that can be differentiated from NSCs. However, there are not many NSCs in the adult brain. Thus, it is imperative to find a way to promote the proliferation and differentiation of endogenous NSCs, or to substitute them for other neuronal sources of NSCs. Bone marrow mesenchymal stem cells (BMSCs) have attracted extensive attention as a new neuron source [8, 9]. At present, BMSCs transplantation has become a routine treatment for various hematological diseases. In this study, BMSCs transplantation was used to explore the plasticity of BMSCs in treating retinal diseases.

Materials and methods

Bone marrow mesenchymal stem cells

The BMSCs were collected from the femur and tibia bone marrow of female SD rats aged 8 to 10 weeks (Azizi, 1998). After anesthesia, the rats were put down to collect their femur and tibia bones. Then, the bones were put into low-glucose DMEM culture medium (containing 10% FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin). After the removal of the epiphysis, the bone marrow was extracted with

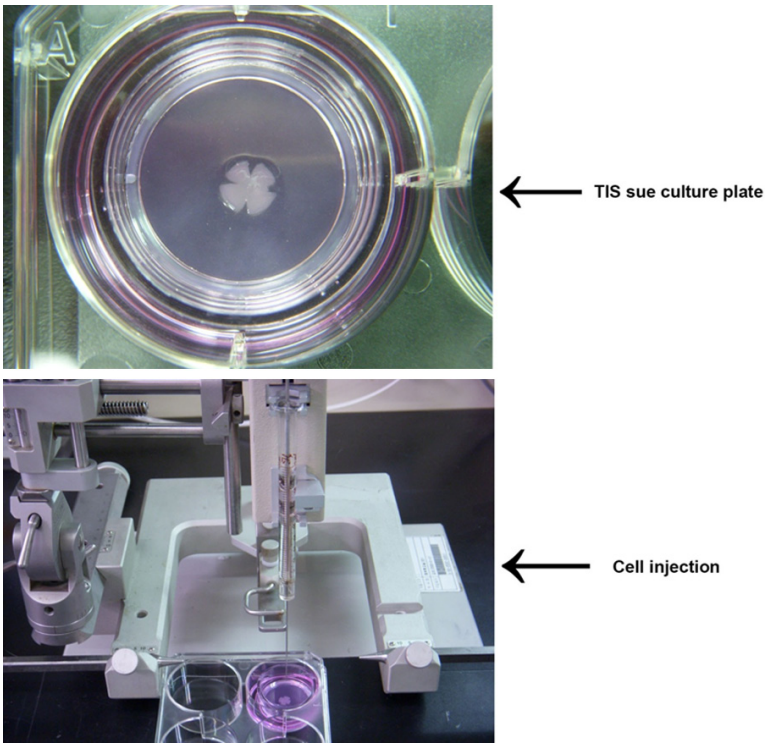

Figure 1. Transplanting stem cell (5×10^5) to cultured retina in vitro with microinjector and microinjection pump.

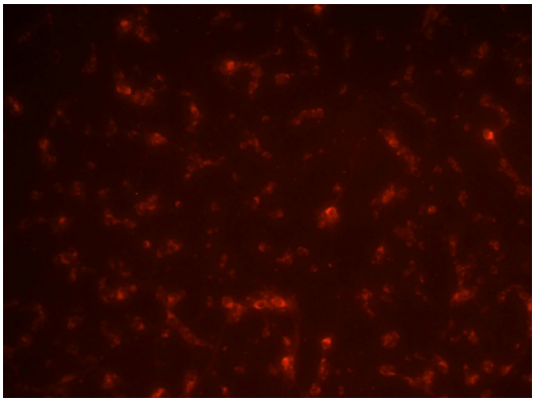

Figure 2. Image of the retina showed stem cells become associated with culture tissue.

a syringe, filtered by 70 μm nylon screen, and centrifuged at 800 g for 5 minutes at room temperature. The cells were suspended, counted, and seeded into the 75 cm^2 cell culture bottle with a density of 10^6 cells/cm^2, and cultured in low-glucose DMEM culture medium (containing 10% FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin) [8]. The culture medium was changed into a fresh medium to remove non-adherent cells after having been cultured for 24 h. Then, it was changed once every 2 to 3 days. When the cells grew into a monolayer, they were digested using 0.25% trypsin, washed with serum-containing culture medium, collected, and centrifuged at 800 g for 5 min. The cells were seeded into a new 75 cm^2 cell culture bottle with a density of 5000-6000 cells/cm^2. The cells were passaged 3 to 4 times. Then the cells were labeled with DiI before transplantation.

Culturing organoretinal tissues and hypoxia damage treatment

The retinal tissue was obtained from Sprague-Dawley rats aged 7 d. Then it was sliced. After anesthesia, the rats were quickly put down. Their eyes were collected to obtain their retinal tissue. The neuroretinal layer and retinal pigment epithelium were separated with tweezers under a microscope. The retinal tissue was cut into slices with a thickness of 400-600 μm by using a high amplitude and low speed vibration slicing machine at 4°C. The slices were cultured in the preparation solution and continuously filled with mixed gas comprised of 95% O_2 and 5% CO_2 (v/v). The retinal slices were then transferred into the transplantable semi-permeable membrane (the diameter of 0.45 μm), in which the ganglion cell layer was placed downwards and cultured in the medium at 35°C, or at room temperature in an incubator with 95% O_2 and 5% CO_2 (v/v). In order to improve the survival quality of the slices, different slice groups were tested under different temperatures and components of the preparation solution and the culture medium. We selected the Ca2-free or low-Ca2 slice preparation solutions to inhibit Ca2-mediated synaptic transmission and neuronal excitotoxicity injury, in which the concentration of Mg2 should reach at least 6 mM. The extracellular capacity of normal neurons accounts for about 20%. There are a large number of glial cells in the neural tissues of mature animals, Cell edema is easily

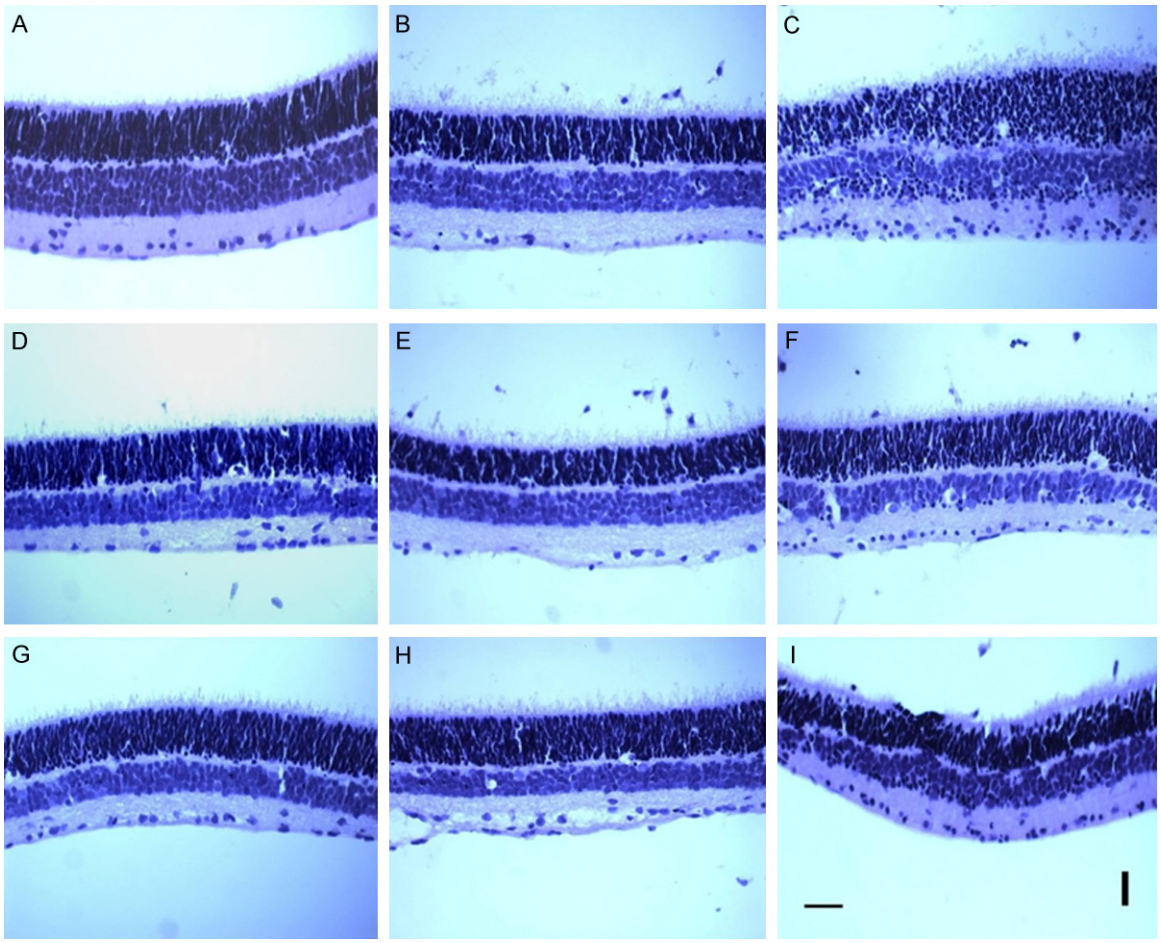

Figure 3. Light micrographs of cultured retinas stained with hematoxylin-eosin. Left column: Control; Middle: Hypoxia; Right: Stem cell injection. A-C: 3 days after each treatment; D-F: 5 days after each; G-I: 7 days after each. Scale bars = 50 μm.

visible during slice preparation. It is helpful to avoid this kind of cell edema by choosing young animals at low temperatures. Studies show that a low temperature preparation solution could delay the decrease of ATP and prolong the survival time of slices in vitro. The metabolic parameters of slices can be improved through pre-treatment of mild hypothermia (29°C-30°C). However, if the slices were treated in the low-temperature culture solution for up to 5 hours, this would reduce the nerve-induced reactions and damage the ultrastructure of the slice. Since 35°C is close to body temperature, and is considered a low temperature, using this temperature could help to maintain the vitality of the section and reduce cell damage. The percentage of edema cells gradually increased in the slices with the increase of their thickness from 300 μm to 700 μm. Obvious histological abnormalities were found in the thick sections of 700 μm. Focal hypoxic tissue appeared in the thin sections of 300 μm. Therefore, the thickness of 400 to 500 μm was most commonly used in this research [10-13]. Hypoxia injury was cultured in a serum-free medium at mixed gas comprised of $N_2/CO_2/H_2$ (85/5/10, v/v/v), 37°C for 1 hour in vitro.

Bone marrow stem cell transplantation

After hypoxia treatment, DiI-labeled BMSCs were transplanted onto the slices and co-cultured. The culture medium was not over the slice, but it was equal to the surface of the inserted semi-permeable membrane under the section. Five retinal tissues were cultured onto each inserted semitransparent membrane and

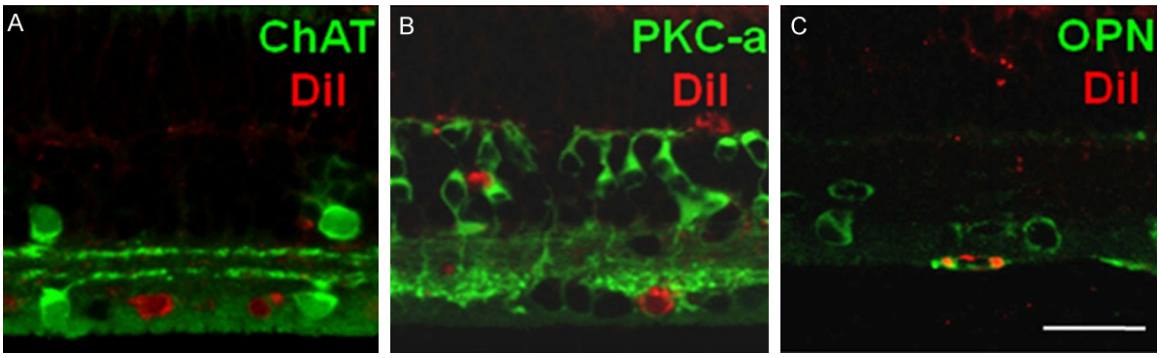

Figure 4. Immunohistochemical visualization of DiI with ChAT (A), PKC-α (B), and OPN (C). Scale bars = 50 μm.

co-cultured after transplanting 5×10^5 BMSCs (**Figure 1**). After culturing them for 3 d, 5 d and 7 d respectively, the sections were fixed and made to observe the structure of retinal tissue and the migration and differentiation of stem cells.

H&E staining and immunohistochemical assay

After 3 d, 5 d, and 7 d of transplantation, all tissues were fixed and made into paraffin sections. H&E staining was conducted to detect the changes of tissue structure. Immunohistochemical assay was used to analyze the changes of protein expression in the tissues, including choline acetyltransferase (ChAT), glutamine synthetase (GS), osteopontin (OPN), and protein kinase C (PKC-α).

The slices were incubated with 3% hydrogen peroxide-methyl alcohol for 10 minutes at room temperature, washed with 0.01 mol/LPBS, blocked using 5% BSA at 37°C for 1 h and incubated with the first antibody (rabbit anti-PKC-α 1:1000, anti-ChAT 1:1000, anti-GS 1:1000, anti-OPN 1:1000, and Santa Cruz) at 4°C overnight. Then, they were washed using 0.01 mol/L PBS, incubated with biotinylated anti-rabbit goat IgG at 37°C for 2 hours, and washed with PBS, finally sealed using glycerol and observed under a microscope [14-17].

Results

Results of co-culture of transplanted cells and retina

On the third day of co-culture of transplanted BMSCs and retina, we found that the cells migrated to each layer of the retina, reaching ganglion cell layer at the deepest level. The BMSCs combined with retinal tissue and established the cell-cell relationship between them. Shown in **Figure 2**, BMSCs labeled with DiI were observed migrating into retinal tissue.

Results of H&E staining

H&E staining results showed that the retinal tissue was seriously damaged after hypoxia treatment (**Figure 3**) and manifested neuronal apoptosis in different degrees. Neuronal synaptic damage resulted in the thinning of the inner reticular layer and the outer reticular layer. This was a significant decrease in the number of neurons in the ganglion cell layer. However, the degree of retinal tissue damage was significantly lower in BMSCs transplantation group compared to that of the control group's, and its thickness was not significantly thinner in each layer. The number of damaged cells was less, and the retinal tissue structure remained better. This suggested that the BMSCs transplantation could inhibit the damage of retinal tissue induced by hypoxia and play a protective role on retinal nerve cells.

Results of immunohistochemical assay

In the results of immunohistochemistry (**Figure 4**), stem cells migrated to the ganglion cell layer, the inner reticular layer, and the inner granule layer to replace the damaged cells.

ChAt: Two ChAT positive areas were found in amakrine IPL of the transverse section of the retina, in which two groups of cells were all monolayer around the ChAT positive areas like a mutual image. The cell bodies of a group of amacrine cells were located in the INL. Their protuberance extended to the IPL and neared the positive area in INL. The other group was

located in GCL, and the protuberance extended to the positive area in GCL. The expression of ChAT was upregulated in early stages, while it gradually reduced over time.

GS: The expression of GS increased in the inner reticular layer and the ganglion cell layer gradually over time. Its expression was the highest in the inner granular layer at 3 d, the highest in the reticular layer at 5 d, and the highest in the reticular and ganglion cell layers at 7 d.

PKC-α and OPN: We found that the transplanted BMSCs migrated to the ganglion cell layer, and the expressions of PKC-α and OPN increased gradually in the inner reticular layer and the ganglion cell layer over time.

Discussion

Stem cell therapy is considered to be an effective treatment for degenerative diseases of the central nervous system, including the retina. In order to improve the therapeutic effect of stem cell transplantation, we analyzed the environmental factors affecting the survival, migration, and differentiation of stem cells. On this basis, we investigated the key factors of the transformation from stem cells to neurons in the ischemic retinal culture system in vitro. Organotype culture is widely used in retinal research. The main advantage of the organic culture system is to maintain the structure of retinal cells and the relationship between cells and nerves, similar to the conditions in vivo [18-20]. Maintenance of cell structure seems to be a key factor in retinal development, differentiation, and survival. BMSCs can differentiate into osteoblasts, cardiomyocytes, and nerve cells. They have many advantages including that they are easy to obtain and that they can expand without losing the potential of multidirectional differentiation in vitro. They also have an easy implantation of exogenous genes. These are all advantages which have garnered more and more positive attention on BMSC's potential clinical application in tissue repair and gene therapy [21-25]. In this study, BMSC was co-cultured with retinal tissue. It was found to have some advantages as a source of allotransplantation.

In this study, the immature rat retina was cultured in vitro. After anoxic injury, BMSCs were well implanted into the cultured retinal tissue and co-cultured. Compared with the control group and the injured control group, the transplanted BMSCs could reduce the retinal damage induced by hypoxia. In the serum-free condition, stem cells were transplanted as neuron-like cells. They were found in the ganglion cell layer after 3 d of transplantation and distributed in each layer of the retina. The transplanted cells could protect the structure of the retina and reduce the injury of nerve cells. The immunohistochemical results showed the cell migration and localization, in which the damaged cells were replaced. These results revealed that the specific differentiation of stem cells depends entirely on the microenvironment where the stem cells are located.

After transplantation of stem cells into the retina, healthy stem cells replaced the lost diseased cells. These transplanted healthy stem cells promoted the survival of peripheral cells. The microenvironmental regulation of the transplanted area can establish effective new synaptic connections and hence improve visual function.

Disclosure of conflict of interest

None.

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