Retinal incorporation and differentiation of mesenchymal stem cells intravitreally injected in the injured retina of rats

Incorporação e diferenciação retiniana de células tronco mesenquimais intravítreas em ratos

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ABSTRACT

Purpose: To evaluate the pattern of retinal integration and differentiation of mesenchymal stem cells (MSCs) injected into the vitreous cavity of rat eyes with retinal injury. **Methods:** Adult rat retinas were submitted to laser damage followed by transplantation of DAPI-labeled BM-MSCs grafts. To assess the integration and differentiation of BM-MSCs in laser-injured retina, host retinas were evaluated 2.4 and 8 weeks after injury/transplantation. **Results:** Our results demonstrated that the grafted cells survived in the retina for at least 8 weeks and almost all BM-MSCs migrated and incorporated into the neural retina, specifically in the outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) while a subset of grafted cells were found in the subretinal space posttransplantation. At 8 weeks immunohistochemical analysis with several retinal specific markers revealed that the majority of the grafted cells expressed rhodopsin, a rod photoreceptor marker, followed by parvalbumin, a marker for bipolar and amacrine cells. A few subsets of cells were able to express a glial marker, glial fibrillary acidic protein. However, grafted cells failed to express pan-cytokeratin, a retinal pigment epithelium marker. **Conclusions:** These results suggest the potential of BM-MSCs to differentiate into retinal neurons. Taken together, these findings might be clinically relevant for future mesenchymal stem cell therapy studies concerning retinal degeneration repair.

Keywords: Mesenchymal stem cells/physiology; Cell survival/drug effects; Retinal degeneration; Retina/injuries; Injections; Vitreous body; Rats

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INTRODUCTION

There are a number of inherited retinal and retinal-neuronal degenerative diseases that can result in blindness, such as retinitis pigmentosa, age-related macular degeneration, glaucoma, and related retinal dystrophies. No effective therapies have yet been developed to prevent or reverse the degenerative processes in these disorders. Retinal transplantation has been considered, but effective neural integration and survival of the differentiated cells have not been reported[1].

Over the past few years, the identification and characterization of stem cells has led the potential use of these cells as a promising alternative to cell replacement therapy. The use of stem cells as a therapeutic option might be a practical approach for treating retinal dystrophies and might help to

restore vision by repopulating the damaged retina and/or by rescuing retinal neurons from further degeneration[2].

From a clinical perspective, mesenchymal stem cells (MSCs) have some potential advantages for retinal transplantation compared to retinal stem cells. Retinal stem cells have been extensively used, but the technical difficulties in obtaining these cells and their limited availability must be overcome prior to the application of these stem cells for human treatment[3]. One great advantage of MSCs is that these cells can be directly obtained from the patient by a simple procedure, thereby eliminating the complications associated with immune rejection of allogenic tissue and infectious diseases. The fact that these cells are easily obtained and propagated in large amounts in culture with potential plasticity and self-renewal capacity also makes them an attractive candidate therapeutic tool for retinal repair by autologous transplantation[4].

In vitro isolation and characterization of MSCs is often based on their adherence, rapid expansion, and expression of specific cell surface antigens, as well as their ability to differentiate into various mesodermal tissues, such as fat, bone, cartilage, and muscle[5-10].

Cultured MSCs can be induced *in vitro* and *in vivo* to differentiate into non-mesenchymal derivatives such as neural cells, a process called stem cell plasticity[11-13].

Bone marrow mesenchymal stem cells (BM-MSCs) differentiate into retinal neural cells *in vivo* and *in vitro*[14], and when implanted at a site of injury in experimental animal models, they demonstrate the ability to migrate to the injury site, initiate tissue repair, and restore function[15-17]. Despite the fact that stem cells integrate into damaged retina layers and differentiate into retinal cells, there has been no evidence of restored functionality or long-term graft survival[18-19].

The purpose of the present study was to evaluate the pattern of retinal integration and differentiation of MSCs injected into the vitreous cavity of eyes with retinal injury.

METHODS

Isolation and Culture of BM-MSCs

Bone marrow was collected from two 2-month-old isogenic Wistar rats by flushing their femurs and tibias with Dulbecco's modified Eagle's medium (DMEM; Gibco). Cells were incubated at 37ºC and 5% CO_2 for 48 h and nonadherent cells were removed. The cells, fibroblast-like in shape, were grown and expanded in flasks for several weeks, and exhibited typical characteristics of MSCs.

Establishment of a laser-injured model

All experiments were performed according to the Ethics Principles in Animal Experimentation (CETEA/UFMG) and approved (certificate number 3/2006).

Wistar rats (n=18) were anesthetized with solution ketamine (40 mg/mL) and xylazine (60 mg/mL), injected intramuscularly. Mydriasis was induced by anesthesia; no eyedrops were used for pupillary dilation. The retinal lesion was induced by an Nd-YAG laser, using an average 0.5 mJ energy. A Goldmann three-mirror lens was used to produce approximately 15 to 20 YAG laser shots around the optic disc. The lesions were all full-thickness retina and choroid disruptions, as observed by deep subretinal hemorrhage and sometimes mild vitreous hemorrhage after the spot was produced. Animals in which the laser caused massive vitreous hemorrhage (n=3) were not used in the study.

BM-MSC labeling and viability

After three passages, bone marrow-derived adherent cells were incubated with 50 µg/mL 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2 for 2 h. BM-MSCs were treated with trypsin (Gibco) for 3 min to generate a single-cell suspension, washed three times with DMEM, resuspended in serum-free DMEM, counted, and stored on ice until transplantation. Fluorescence from DAPI labeling of BM-MSCs and cell viability were evaluated at 1, 2, 3, 4, and 8 weeks after cell labeling.

Intravitreal transplantation of MSCs

Rats (10 - 12-week-old Wistar SHR rats; n=18) were anesthetized by intramuscular injection with solution ketamine (40 mg/mL) and xylazine (60 mg/mL). A cell suspension (20 µl) containing approximately $4x10^5$ DAPI - labeled BM-MSCs was slowly injected into the vitreous cavity via the pars plana using a 30 gauge needle 24 h after the retinal lesion was induced by the laser in 9 animals (both eyes). Three groups of animals were studied: 1) rats with retinal injury and injection of BM-MSCs (n=9); 2) rats with retinal injury without injection of BM-MSCs (n=9); 3) rats without retinal injury with injection of BM-MSCs (n=9).

Tissue sectioning

The animals were sacrificed 2, 4, and 8 weeks following transplantation. The eyeball was enucleated by performing a 360º limbal peritomy, isolating the extraocular muscles, and dissecting the optic nerve at the apex of the orbit. The eyes were either processed freshly (embedded in OCT compound and freshly sectioned) or fixed in Omnifix (FR Chemical Inc, Mount Vernon, NY). For both processes, slides were stained with hematoxylin/eosin, and visualized under a compound microscope to evaluate retina morphology.

Immunohistochemical detection

All Paraplast-Plus embedded blocks were cut, deparaffinized, rehydrated, and subjected to immunostaining with several antibodies specific for the retinal cells. Following blocking for 1 h at room temperature in phosphate buffered saline (0.15 M PBS), 1% bovine serum albumin, and 2% normal bovine serum, ocular tissue sections were exposed to primary antibodies (Table 1) overnight at 4ºC in a moist

chamber. Control sections were treated identically, but the primary antibody was omitted. Sections were then washed three times for 5 min each in 0.15 M PBS. Secondary antibody (goat anti-rabbit and anti-mouse labeled with Alexa Fluor 488 (1:500, Molecular Probes, Invitrogen) was then applied for 1 h at room temperature. Sections were washed three times for 5 min each in 0.15 M PBS. The slides were viewed under a fluorescence microscope (Olympus®, Optical sectioning microscope attached to an Axioplan imaging Apotome® apparatus, Zeiss®, Germany). Colocalization of Alexa 488 - labeled primary antibodies and DAPI was assessed by superimposing separate digital images of each fluorochrome.

RESULTS

BM-MSCs characteristics and transplantation

After three passages in culture, the isolated cell population became homogeneous, showing a monolayer consisting of adherent cells displaying further traits of MSCs, including a typical fibroblast-like morphology and increased proliferation (Figure 1 Left and Right). Adhesion to the culture dish also served as a criterion to distinguish MSCs from free-floating hematopoietic cells.

Incorporation and distribution of the grafted cells

To verify the fate of BM-MSCs grafted in the laser-injured rat retina, the cells were prelabeled with DAPI and injected into the vitreous space. Retinal damage has an important role in the incorporation of transplanted cells in the retina, due to the high expression of chemoattractants by injured retinal pigment epithelium (RPE). To determine the distribution of BM-MSCs in the retina, we examined the tissue 2, 4, and 8 weeks after the grafting procedure (Figure 2). The transplanted cells were incorporated in a time-dependent manner. Survival of the grafted cells was substantial in all transplanted eyes at all evaluated points. At 2 weeks, the majority of the grafted cells remained in the vitreous space (Figure 2, top, right) and at 4 weeks the cells had migrated and incorporated into the host neural retina (Figure 2, bottom left). At 8 weeks, the grafted cells were almost fully incorporated into the host neural retina; BM-MSCs were present in the outer nuclear layer, inner nuclear layer, and the ganglion cell layer (Figure 2, bottom, right).

Immunohistochemical analysis and fluorescence microscopy

Immunohistochemistry was performed on sections in which BM-MSCs were incorporated in the injured retina at 8 weeks after transplantation and in the control group to examine whether the grafted cells distributed into the retinal layers expressed retinal cell type-specific markers. The cell-type markers used were rhodopsin for rod photoreceptor cells, glial fibrillary acidic protein (GFAP) for Muller cells and astrocytes, parvalbumin for bipolar and amacrine cells, and pancytokeratin for RPE (Table 1).

Most of the DAPI-labeled cells, which we presumed to be the grafted BM-MSCs, incorporated into the outer nuclear layer (ONL) and expressed rhodopsin (Figure 3, top of the right panel), and parvalbumin (Figure 3, top middle of the right panel), suggesting their potential to differentiate into rod photoreceptors and amacrine or bipolar cells, respectively. A small subset of labeled cells in the inner retina was also labeled for the glial marker GFAP (Figure 3, bottom, middle of the right panel), suggesting that these cells possess the ability to differentiate into astrocytes and Muller cells. Some DAPI-labeled cells were also observed in the subretinal space, but they did not express any retina-associated markers at any point (Figure 3, top and middle bottom of the right panel). This finding suggests that these cells might provide a local milieu that somehow evolves with the incorporation and differentiation of the grafted cells. In the control group sections (where the injured retina did not receive BM-MSC transplants), the retina-specific markers were evident and no DAPI-labeled cells were observed, ruling out the possibility of auto-fluorescence of the retinal cells (Figure 3, left panel). There was also no co-localization of DAPI-labeled cells with the RPE-specific marker (Figure 3, bottom, right panel), suggesting that the grafted cells did not differentiate into RPE cells.

DISCUSSION

There are several sight-threatening retinal disorders for which treatment is not yet available or produces poor results. In some of these diseases, retinal degeneration occurs early in life and might be quite rapid, whereas in other disorders, retinal degeneration begins later and progresses very slowly. There are some possible sources for the repair of retinal dege-

Table 1. Primary antibodies for immunohistochemistry

	Antigen	Clone	Species	Supplier	Dilution
Immunohistochemistry	Rhodopsin	[RET-P1]	Mouse monoclonal	Abcam	1:16
	GFAP (Glial fibrillary acidic protein)		Rabbit	Abcam	1:1000
	Parvalbumin		Rabbit	Abcam	1:1000
	Pan-cytokeratin	[C-11]	Mouse monoclonal	Abcam	1:250

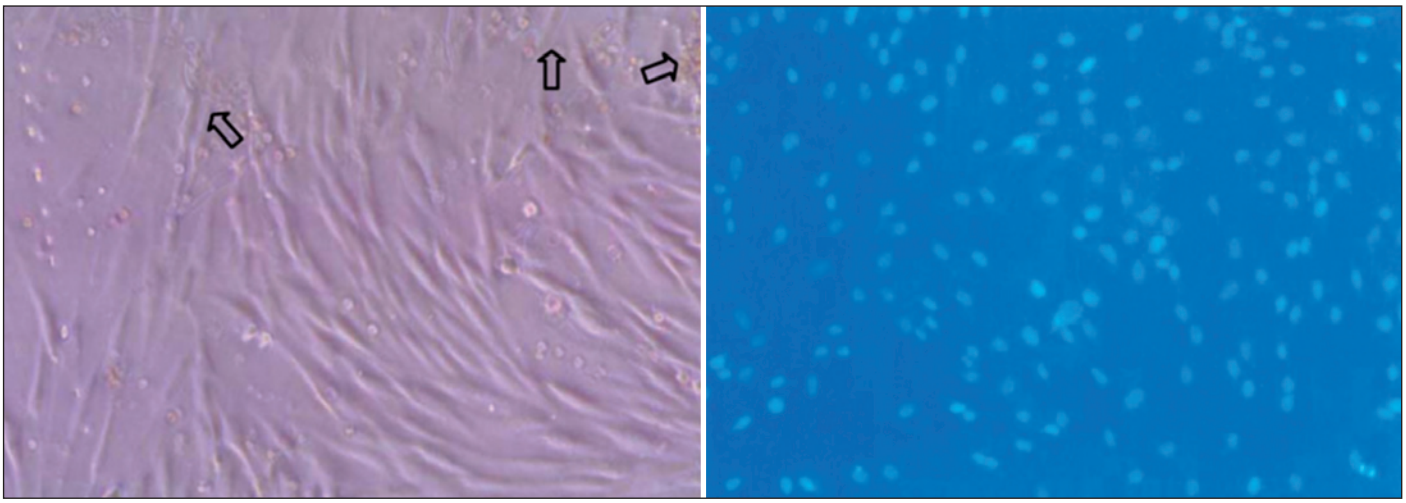

Figure 1 - Morphologic analysis of cultivated and DAPI-labeled rat bone marrow mesenchymal stem cells (BM-MSCs). (Left) Phase contrast microscopy of rat BM-MSCs in the cell culture dish at passage 3, showing a stretched fibroblastic phenotype. Arrows shows the presence of round-shaped erythrocytes and nonadherent cells. After expansion to the third passage, a monolayer of adherent, fibroblast-like cells were labeled with DAPI. (Right) Fluorescence microscopy of adherent, fibroblast-like cells labeled with DAPI. Original magnification X150.

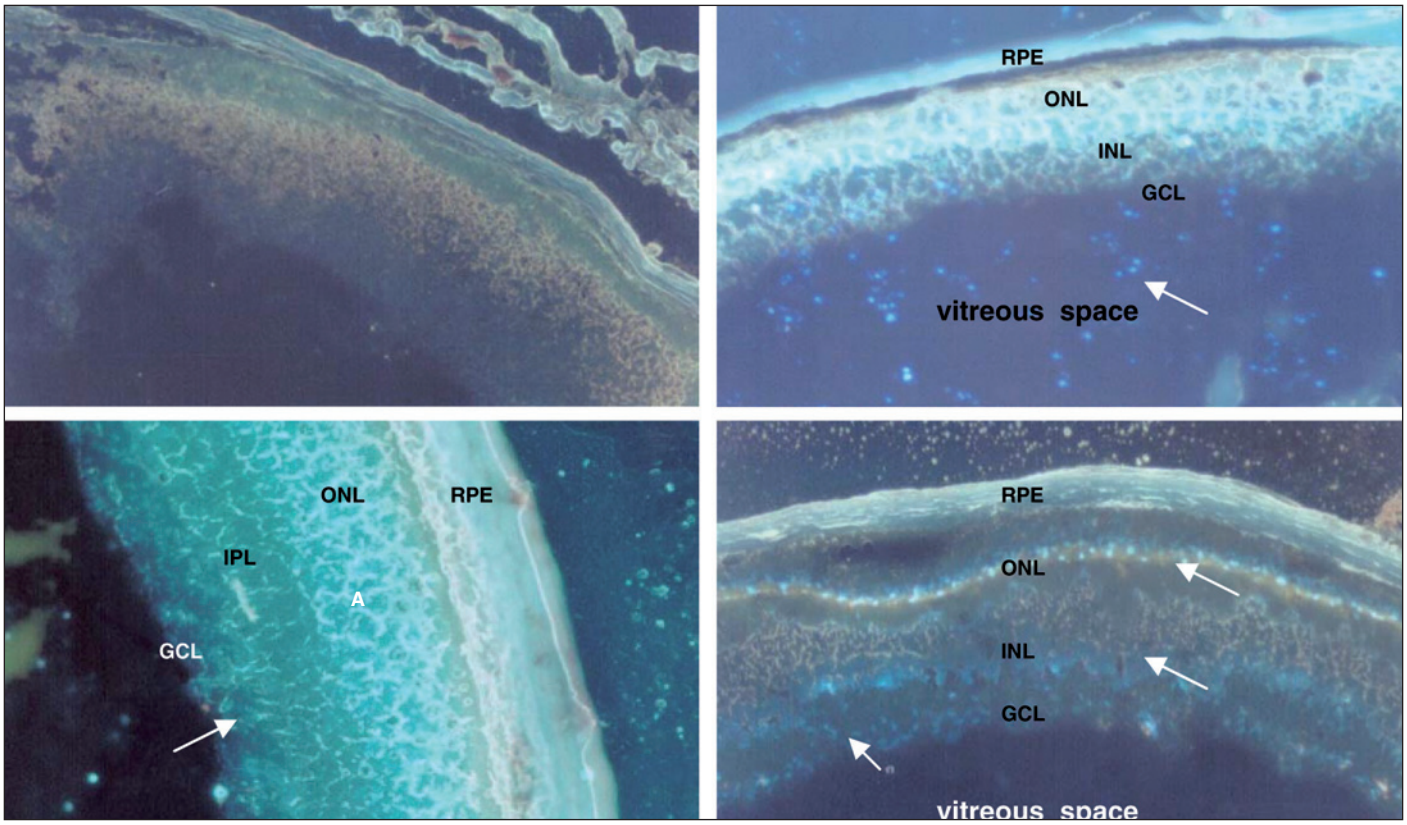

Figure 2 - DAPI-labeled BM-MSCs transplanted into the intravitreal space in rats with laser-induced damage to the retina. Distribution of the grafted cells, (top rigth) 2 weeks after transplantation, most of the grafted cells were still in the vitreous space, (bottom left) 4 weeks after transplantation, a few grafted cells incorporated into the neural retina, (bottom right) 8 weeks after transplantation, most of the grafted cells were observed in the ONL, INL, and GCL of the neural retina, (top left) control group - injured retina without BM-MSC transplantation. White arrows shows the location of the grafted cells after transplantation. Original magnification X150. RPE= retinal pigment epithelium; ONL= outer nuclear layer; INL= inner nuclear layer; GCL= ganglion cell layer

neration. A current therapeutic approach to neovascular eye diseases involves the application of angiostatic or antiproliferative agents to the eye. A cell-based therapy, however, should be explored, because, in contrast to inhibiting angiogenesis with small molecules or recombinant factors, a cell-based approach might enable the cell to adapt and respond to

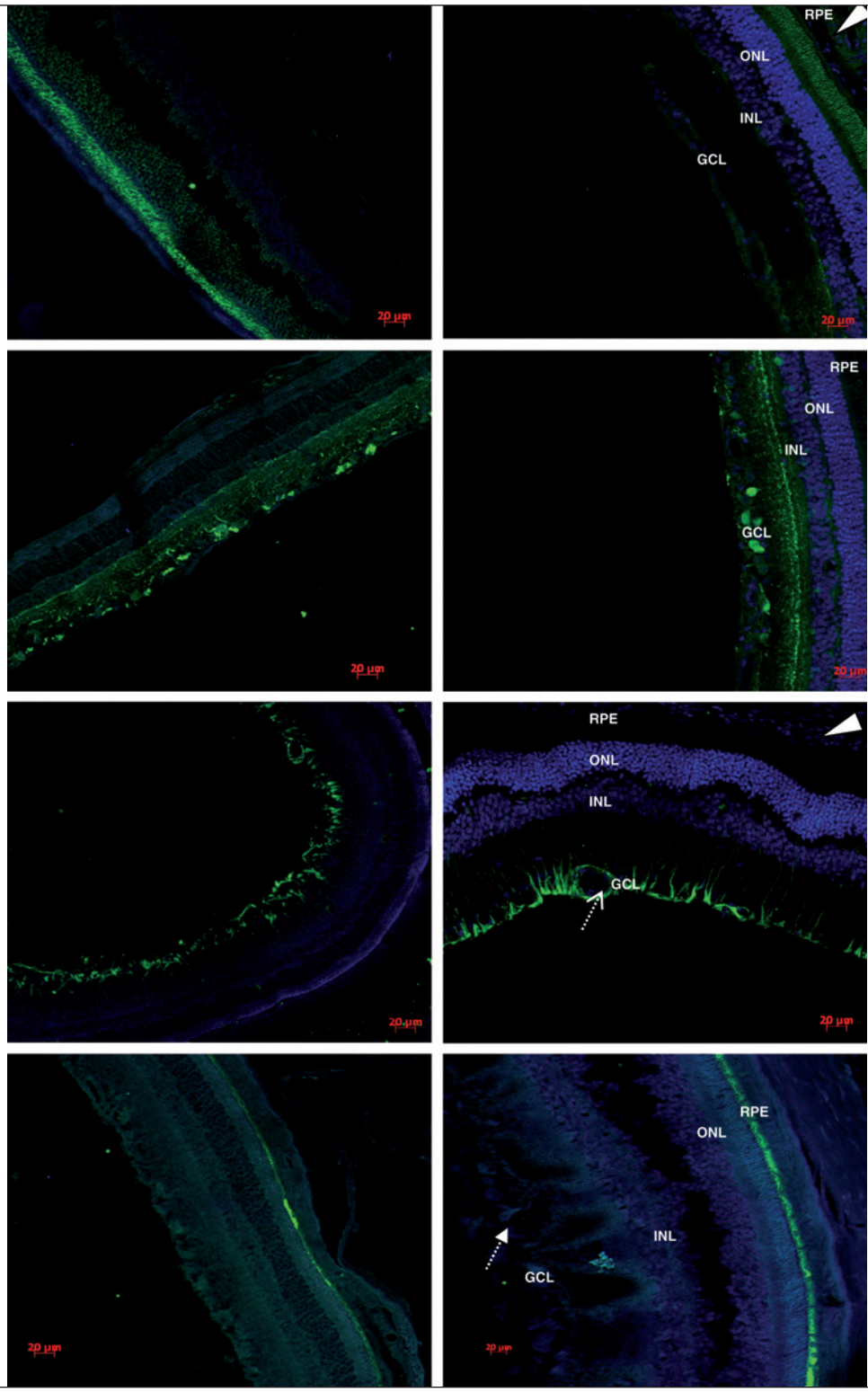

Figure 3 - Immunohistochemical detection of DAPI-labeled BM-MSCs in injured retinal sections 8 weeks after intravitreal transplantation. The left panel of each row shows immunoreactivity for a particular marker in retinal sections with laser-induced injury without BM-MSC treatment (control). The right panel of each row shows immunoreactivity for a particular marker (green) in retinal sections with laser-induced injury and BM-MSC treatment. Eight weeks after transplantation, the grafted cells labeled with DAPI (blue) co-expressed the rod photoreceptor marker (green) (top) rhodopsin, the bipolar and amacrine cell marker (green) (top middle) parvalbumin and the Muller cell and astrocyte marker (green) (bottom middle) GFAP. No grafted cells coexpressed the RPE marker (green) (bottom) pan-cytokeratin. Note that only a small subset of grafted cells was GFAP-immunoreactive (dashed arrows). Arrowheads in the right panel (top) and (bottom middle) indicate the presence of grafted cells (blue) in the subretinal space. Micrographs of the control group did not show any nonspecific labeling or fluorescence expression. Scale bars, 20 µm. RPE= retinal pigment epithelium; ONL= outer nuclear layer; INL= inner nuclear layer; GCL= ganglion cell layer.

a changing environment. Cell-based therapy likely involves numerous factors produced by the cell that can be appropriately modulated in response to changing conditions[20]. It was demonstrated that MSCs are able to secrete neurotrophic factors that promote neural cell survival[21].

The multipotentiality of BM-MSCs as well as their easy isolation and culture properties and their high expansion potential makes these cells an ideal source for autologous transplantation aimed at a cell-based therapy for retinal degeneration repair[2].

Several studies have demonstrated the survival, incorporation, and morphologic integration of transplanted neural stem cells in injured retinas[22-24]. Few studies, however, have examined the potential of BM-MSCs to regenerate damaged retinas[25] injecting stem cell-enriched bone marrow cells into injured rat eyes and observed that the cells were incorporated into the host retina and that some of the CD45+ cells differentiated into retinal neural cells *in vivo.* MSCs can also be induced to differentiate into photoreceptors *in vitro* and *in vivo*[14].

Also, a recent study compared MSCs and retinal progenitor cells to evaluate their potential as a source for retinal transplantation[26]. Both cell types expressed neuronal markers *in vitro* but some MSCs differentiated into cells that resembled microglia rather than neural cells. These findings suggested that retinal progenitor cells are the best choice for retinal transplantation studies, but MSCs remain an attractive candidate as a therapeutic tool for retinal repair in autologous transplantation.

In the present study, we injected BM-MSCs intravitreally into the rat eye 24 h after an Nd-YAG laser injury in the retina and observed that the grafted cells dispersed into the host retina and assumed different patterns of distribution. Specific antibodies were used to determine the ability of grafted MSCs to morphologically differentiate within the environment of transplanted eyes. Co-localization of DAPI with one of the specific phenotypic antibody markers was used to evaluate neural differentiation of the grafted cells. The grafted BM-MSCs incorporated into the host neural retina and expressed GFAP, rhodopsin, and parvalbumin, suggesting neural differentiation.

The presence of a subset of cells in the subretinal space suggests that these cells are accelerating the regeneration process of the retina. The stimulatory effect of BM-MSCs is related to the secretion of various neurotrophic factors that activate compensatory processes in abnormal tissues[27-29]. Also, BM-MSCs produce several matrix molecules and have a great potential to repair damage resulting from injury, ischemia, and normal aging[30].

In addition to the used cell type, another issue to be investigated is the preferential method of transplantation of the stem cells close to the retina. There are two methods of transplantation, subretinal and intravitreal, that are widely described in the literature and both seem to be effective. The subretinal implantation of stem cells is a more demanding and complex procedure. Intravitreal injection is a more popular and a much less invasive procedure. No apparent differences in the efficacy of these cell engrafting procedures are reported in the literature, thus intravitreal injection is the best choice because it is less invasive. The vitreous body anatomy of the rat eye, however, should also be considered. The vitreous cavity in the rat eye is crescent moon-shaped, which brings the injected cells closer to the retinal surface, perhaps allowing easier access of the cells to the retinal layers.

A comparison of the incorporation of cells between the injured and non-injured groups suggested that the extent of the lesion had a role in the engrafting of the cells. The study demonstrated that the cells incorporated and differentiated in the injured group, but not in the non-injured group, as previously reported by some authors. Migration and integration of the transplanted cells into the host tissue is probably induced by its injury. According to Chacko, the widespread migration and incorporation of neural stem cells was observed only in retina that was either diseased[24] or traumatized[23]. Those injuries could provide a local milieu (interleukins, chemotaxins, inflammatory and growth factors, etc), that is responsible for the migration and incorporation of exogenous ocular stem cells. It was recently reported that damaged RPE secretes cytokines that serve as chemoattractants for BM-MSCs by regulating the homing and migration of these cells to the injury site[17].

The use of DAPI as a cell tracer can become a problem in transplant experiments, due to its considerable diffusion and uptake by host retinal cells. To overcome this problem, there are now some ongoing experiments, in our laboratory, using MSCs labeled with GFP.

These *in vitro* studies are essential to understand the process of stem cell-based therapies and allow for the evaluation of the nature of the factors responsible for the homing of these cells as well as the conditions that promote integration and differentiation of the grafted cells into the host retina. Once these issues are clear, there is a great potential for the use of stem cells in clinical therapy, but further studies are needed to ensure their safe and effective transplantation.

CONCLUSIONS

The present study demonstrated that MSCs can incorporate and differentiate into specific cellular components of the retina after laser-induced tissue injury.

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RESUMO

Objetivo: Avaliar o padrão de integração e diferenciação retiniana de células tronco mesenquimais (CTM) injetadas na cavidade vítrea de ratos portadores de lesões retinianas. **Métodos:** Ratos Wistar adultos foram submetidos a múltiplas lesões retinianas utilizando-se YAG laser e injeção intravítrea de células tronco mesenquimais. A fim de se avaliar a integração e diferenciação retiniana, o tecido retiniano lesado pelo YAG laser / tratado pelas células tronco, foi avaliado 2, 4 e 8 semanas após a lesão. **Resultados:** As células injetadas na cavidade vítrea sobreviveram na retina por pelo menos 8 semanas e quase todas células tronco mesenquimais migraram e incorporaram-se na retina neural, especificamente nas camadas nucleares externa e interna e camada de células ganglionares. Uma pequena quantidade de células foi encontrada no espaço sub-retiniano. A análise imuno-histoquímica de 8 semanas mostrou que a maioria das células injetadas expressou rodopsina (marcador para fotorreceptores), parvalbumina (marcador para células bipolares e amácrinas), GFAP (marcador de células gliais). As células injetadas não expressaram a pancitoqueratina, que é a marcadora de células do epitélio pigmentar da retina. **Conclusões:** Ocorre aparente diferenciação e incorporação de células tronco mesenquimais na retina de ratos após injeção intravitrea destas células.

Descritores: Células-tronco mesenquimais/fisiologia; Sobrevivência celular/efeitos de droga; Degeneração retiniana; Retina/lesões; Injeções; Corpo vítreo; Ratos

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