Monitoring of stem cells from adipose tissue injected via retrobulbar next to previously injured optic nerve of rabbits

Monitorização de células-tronco mesenquimais injetadas via retrobulbar próximas ao nervo óptico lesados de coelhos

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Abstract

Obtective: *To verify the presence of mesenchymal stem cells (MSC) in the area close to the optic nerve of previously injured with absolute alcohol.* ***Methods:*** *Twelve New Zealand breed rabbits were divided into two groups, and after sedation, each eye of the animal received a retrobulbar injection of 1 ml of absolute ethanol in one eye, and 1 ml of physiological solution 0.9 % (PS) in the contralateral eye. After 15 days all eyes of animals belonging to group A, received via retrobulbar a solution containing MSCs from human adipose tissue (AT) and previously marked with Qdots, while all eyes of animals from group B received solution containing PBS.* ***Results:*** *The presence of MSC was observed in 100% of the eyes of the animals of group A and the more central areas near and into the optic nerve.* ***Conclusion:*** *The results suggest that the appointment of MSC with Qdots allowed their follow-up applied in the region and in the inner areas of the optic nerve. The MSC permanence after 15 days of application around the optic nerve suggests the feasibility and possible involvement of the same during the damaged tissue regeneration process. Under the conditions of this study, the route of retrobulbar application and the presence of the stem cells to the central areas of the optic nerves in animals of group A, suggests that this might be an effective approach for MSCs in regeneration process of optic neuropathies.*

Keywords: *Mesenchymal stem cells; Optic nerve; Neuropathy; Qdots; Rabbits*

Resumo

Objetivo: Verificar a presença das células-tronco mesenquimais (MSC) na área próxima ao nervo óptico de coelhos previamente lesado com álcool absoluto. **Métodos:** Os 12 coelhos da raça Nova Zelândia foram distribuídos em 2 lotes. Após sedação, cada olho do animal recebeu uma injeção retrobulbar de 1 ml de álcool absoluto em um dos olhos e de 1 ml de solução fisiológica 0,9% (SF) no olho contralateral. Após 15 dias deste procedimento inicial todos os olhos dos animais pertencentes ao lote A, receberam via retrobulbar, uma solução contendo MSC de tecido adiposo humano e previamente marcadas com Qdots,. Todos os olhos dos animais do lote B receberam solução PBS. **Resultados:** Após 15 dias desta última aplicação os animais foram sacrificados e as lâminas foram analisadas. A presença das MSC foi observada em 100% dos olhos dos animais do lote A. **Conclusão:** Os resultados sugerem que a marcação prévia das MSC com Qdots permitiu o acompanhamento das mesmas na região aplicada e em áreas mais internas do nervo óptico. A permanência de MSC após 15 dias de aplicação ao redor do nervo óptico sugere a viabilidade e possível participação das mesmas no processo de regeneração do tecido lesado. Nas condições deste estudo, a via de aplicação retrobulbar permitiu a mobilização das células tronco do local de aplicação até áreas centrais dos nervos ópticos nos animais do lote A, sugerindo que esta poderá ser uma via de acesso eficaz para as MSC no processo de regeneração de neuropatias ópticas.

Keywords: Células tronco mesenquimais; Nervo óptico; Neuropatia; retrobulbar; Qdots; Coelhos

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Introduction

According to the WHO data released in 1990, there were about 38 million blind people in the world, and 110 million people at risk of blindness. By 2020, blindness could mutilate about 76 million people worldwide, mainly due to optic nerve atrophy. [1-4] Definitive neuropathy may result from diseases or complications such as chronic papilledema caused by brain tumors (not resectable or after resection), optic nerve hemorrhage, acute retinal necrosis syndrome, central retinal vein occlusion, nonarteritic ischemic optic neuropathy, glaucoma, among others.[5-7]

Glaucoma neuropathy is already considered the third leading cause of irreversible blindness in the world.[8-10] Better knowledge of the pathophysiology and especially the molecular chemistry of this disease have led to the emergence of promising new perspectives of treatment: therapeutic and regenerative. This innovative perspective of regenerative medicine along with several studies have shown that stem cells have a great capacity for self-generation, proliferation, expansion and differentiation, and may even reconstruct damaged tissues and even form a new organ.[11-13]

The immunomodulation capacity coupled with the lack of expression of histocompatibility antigens, makes mesenchymal stem cells (MSC) - among the other stem cell lineages - probably be ideal for use in regenerative medicine techniques.[14,15]

The main source of stem cells is bone marrow, but they can be obtained from other tissues such as cord blood, fetal liver, amniotic fluid, dental pulp, and adipose tissue. Although the use of cord blood has been facilitated by the adoption of a public policy to encourage cord blood banks in recent years, another source of MSC collection which tends to be very useful is from adipose tissue because of the great ease to collect during liposuction procedures.[16-19]

Stem cells from adipose tissue (MSC) are able to differentiate into cells originating from the mesoderm lineage (bone, cartilage and tendons) as well as those from endo and ectoderm lineages, and may originate cardiomyocytes, neurons and liver cells, among others. [20-23]

Characterization studies have shown that MSCs do not express histocompatibility antigens, HLA, class II of CD80, CD86 and CD40 costimulatory molecules, nor markers ofthe hematopoietic lineage CD45, CD3, CD31. They express HLA class I in small amounts, which decreases host rejection rates. They also have immunomodulation capacity. Depending on the stimulus, they immunosuppress or stimulate.

MSCs control the secretion of antiproliferative factors, inhibiting the T lymphocyte proliferation and cytotoxic action. They also act on natural killer (NK) lymphocytes and prevent dendritic cell maturation and function. In the presence of Interferon gamma, MSCs eventually release intranuclear HLA class I antigens to the surface, enhancing immunogenicity.[18, 24,25] Due to their ability to immunomodulate the immune response and regenerate the tissue, some studies with animal modelsareevaluating the potential of these cells in the treatment of autoimmune diseases such as multiple sclerosis, diabetes, systemic lupus erythematosus, among others.[18]

Mesenchymal cells, when applied intravenously in mouse experiments, differ in neuronal cells in the central nervous system. [26-28] Sasaki et al. [29] showed that in mice MSC are capable of differentiating into myelin fibers and repairing the spinal cord, i.e., they confirmed that under appropriate conditions these cells differentiate into neurons.[14,15] Still in the field of Ophthalmology, there are reports in the literature of use of brain neuronal cells from newborn or embryonic animals for retinal transplants.[14,15]

The feasibility of developing stem cell therapy, especially MSCs, together with the use of neuronal differentiation-inducing factors and the confirmation of their penetration into injured tissues has encouraged the present study.

Objective

Confirm the presence of MSC from adipose tissue in previously injured rabbit optic nerves. Analyze the effectiveness of the retrobulbar application of MSCs and their participation in the regeneration of the previously injured optic nerve.

Methods

Animal experiments were carried out at the Experimental Surgery and Medicine Center of the School of Medical Sciences at Universidade Estadual de Campinas (UNICAMP). The parts and the material intended for immunohistochemical study were prepared at the Experimental Laboratory of Pathological Anatomy of the School of Medical Sciences at UNICAMP following the histological technique for paraffin sectioning. Extraction, differentiation, culture and expansion, and immunophenotypic analysis of mesenchymal cells were carried out at the Cell Biology Laboratory of the blood center at UNICAMP. The immunofluorescence slides were analised and photographed at Instituto Nacional de Fotônica Aplicada à Biologia Celular -INFABIC and the Cell Biology Laboratory of the blood center at UNICAMP

1. Sources of Mesenchymal Cell Obtainment

MSCs were obtained from human adipose tissue from patients undergoing liposuction surgery under the effect of general anesthesia at the Clinic Hospital of the School of Medical Sciences at UNICAMP. The procedures were approved by the Research Ethics Committee of the School of Medical Sciences - UNICAMP (case CEP-No. 838/2008). After clarifying the nature of the study, all patients undergoing liposuction who agreed to donate signed the Informed Consent Form. Patients aged 25 to 50 years were selected, with diabetic and hypertensive patients being excluded.

2. Obtaining Mesenchymal Cells

a. Adipose Tissue

After liposuction, the material collected was submerged in sterile PBS-Phosphate Buffer Solution. The adipose tissue obtained was then dissected into small fragments and washed with PBS in centrifugation 1500 rpm for 8 minutes. The fragments were immersed in an enzyme solution with collagenase type I (1.5mg/mL), 25mM Hepes and bovine serum albumin (BSA-20mg/mL) for 30-90 minutes at 37°C under continuous stirring until dissolution of the fatty tissue. The enzymatic reaction was stopped by the addition of an equal volume of low glucose DMEM (Dulbecco's Modified Eagle's Médium/Gibco, Rockville) with 10% fetal bovine serum (FBS) and centrifuged at rotation of 1500 rpm for 10 minutes. The cell pellet was resuspended in erythrocyte lysis buffer (pH 7.3) for 10 minutes, and then washed in 40 ml of cold PBS at 1200g for 10min. Finally, the cells obtained were resuspended in DMEM-low glucose culture medium with 10% FBS and cultured at a density of 1.5x105cells/mL. Cells were kept

in an incubator at 37°C in 5% CO2 and 95% humidity. After 2-3 days of incubation, cells were washed with culture medium to remove dead or nonadherent cells.

3. Expansion and culture of mesenchymal stem cells

Cells adhered to the culture plate were incubated for 5-7 days in incubator at 37°C in 5% CO2 and 95% humidity with DMEN-low glucose / 10% FBS culture medium for proliferation. When they reached confluence of 70-80%, they were trypsinized (Trypsin, Gibco, Rockville), counted (Neubauer Chamber), and distributed at a concentration of approximately 4x10 3 cells/cm2 in DMEM-LG / 10% FSB culture medium with antibiotic. After the fourth pass, adherent cells were characterized as mesenchymal adipose tissue stem cells (Figure 1).

4. Immunophenotyping of undifferentiated mesenchymal cells by flow cytometry

After the fourth pass, the adherent cells were collected, washed and resuspended in 50 µL of wash solution (PBS1X without Ca2+ Mg2+, 3% FCS, 10mM HEPES pH 7.2). Then the following antibodies were added: anti-CD73, anti-CD90, anti-CD105, anti-CD3, anti-CD14, anti-CD45 and anti-IGg (positive control). Cells were washed twice with wash solution to remove excess antibody, and centrifuged at 220g for 5 minutes. Ten thousand events were acquired with a FACS Calibur (Becton-Dickinson, CA, USA), and analyzed with Cell Quest Software (Becton-Dickinson, San Jose, CA, USA). Nonspecific labeling was verified from the fluorescence intensity of the corresponding isotypic control, and subtracted from the corresponding positive population percentage.

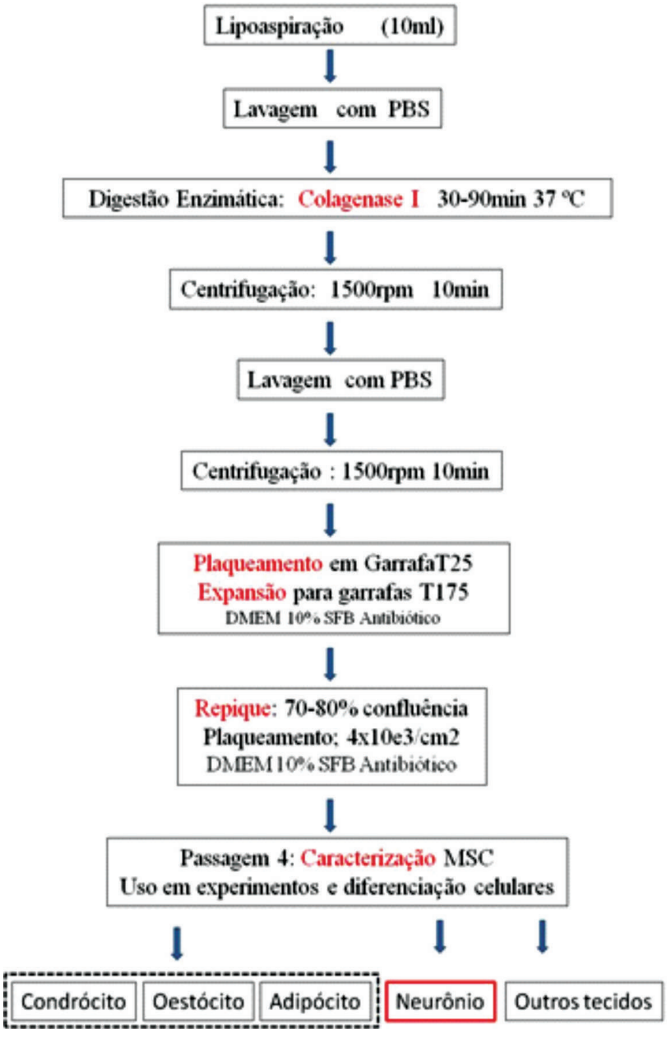

Figure 1: Obtaining mesenchymal stem cells from adipose tissue.

5. Real-time PCR (RT-PCR) gene expression analysis

Total RNA was extracted from undifferentiated mesenchymal cells (control) and mesenchymal cells at different stages of the differentiation process using the Neasy® Micro Kit R (Qiagen) following the guidelines described by the manufacturer. RNA samples were treated with DNAse I enzyme to eliminate genomic DNA contamination, and quantified by spectrophotometry at 260nm. The amount of RNA extracted was evaluated by 1.2% agarose gel electrophoresis stained with ethidium bromide. The RNA samples treated were transcribed into cDNA using the SuperScript III enzyme, and also quantified by spectrophotometry at 260nm. Gene expression was analyzed by real-time PCR technique on ABI 5700 equipment using SYBRGreen reagent. The genes Collagen II, Agrecane and SOX 9 were analyzed for the characterization of chondrocyte differentiation; Osteocalcin and Osteopontin for the differentiation into osteocytes; and FABP4, PPARγ and LPL for the adipocyte differentiation. Regarding neuronal differentiation, the expression of the genes Nestina, beta III tubulin, Nkx6.1 and Ngn3 was analyzed.

6. Confocal Laser Scanning Microscopy for Cell Phenotype Study

A cell sample was cultured and subjected to neuronal differentiation on glass coverslips treated with poly-L-isine. Cells were collected 8 days after treatment, fixed with paraformaldehyde in phosphate buffer for 15 min at room temperature, and washed in PBS. Primary antibodies were incubated for 18h at 4°C in PBS solution containing Triton X-100 and skimmed milk. After incubation with primary antibodies, cells were incubated with fluorescein or rhodamine conjugated secondary antibody for 2 hours at room temperature, and the coverslips were mounted and evaluated under a confocal microscope. Primary antibodies used were anti-nestin (goat polyclonal IgG); beta III tubulin (mouse monoclonal IgG); anti-synaptophysine (rabbit polyclonal IgG).

7. Labeling for Cell Tracking in vivo

For screening MSCs in vivo, cells were labeled with Qdots, the Qtracker Cell Labeling Kit (Invitrogen). The cells were trypsinized (Ginbco), suspended in culture medium (DMEM-LG / 10% FBS), and counted. The protocol from this point on follows the product manual. Briefly, a 10mM labeling solution was prepared by mixing 1µl of components A and B (Kit) at room temperature for 5 minutes. 0.2ml growth culture medium (DMEM-LG / 10% FBS) was added and mixed under vigorous stirring for 30 seconds. The solution containing 1x106 cells was added to the labeling solution, being gently stirred every 5 to 10 minutes, and incubated at 37°C for 45-60 minutes. After this period the cells were washed twice with growth culture medium, and subjected to in vitro marking permanence test for later generations, or were washed with PBS for subsequent injection into the animal.

ANIMALS

Twelve female rabbits aged 2 to 4 months of New Zealand breed weighing between 800 and 1900 grams (average of 1135.60 grams) from Granja RG were used. Before being included in the study, all animals were kept in a constant temperature environment (21ºC). The animals presented good general health, free from ecto and endoparasites. Surgical procedures at the Experimental Medicine Center were carried out after the rabbits were found to be in perfect health, in accordance with the ARVO Association for Research in Vision and Ophthalmology guidelines and the rules for the use of animals in scientific experiments stipulated by the Brazilian College of Animal Experimentation (COBEA - Colégio Brasileiro de Experimentação Animal). The protocol of the present study was submitted to the Ethics Council of the Center for Experimental Medicine and Surgery, and approved under protocol CEEA IB 15891. All rabbits were weighed, then placed in individual cages, and marked with an identification number (11 to 22). For specific identification of each animal, hydrographic pen was used, with both ears being inscribed with the number of the corresponding cage. Female rabbits 11 to 16 were included in batch A, and 17 to 22 in batch B. Next, the female rabbits were anesthetized with a mixture of ketamine at a dose of 50 mg.kg-1 and Rompun at a dose of 5 mg.kg-1 applied intramuscularly. Topical anesthesia was performed by instilling 0.5% tetracaine (1 drop/eye) after irrigation of the eyes with 0.9% sodium chloride solution.

In this double-blind study in which the operator was unaware of the type of solution employed for treatment, absolute alcohol and SF solutions were previously prepared in a 10 ml syringe by a professional who did not participate in the surgical procedures nor laboratory analysis. Both solutions (SF and alcohol) were randomly aspirated into similar syringes identified with numbers 1 and 2. For the experiment, 1 ml of solution in syringe 1 was applied to the right eye, and 1 ml of solution in syringe 2 to the left eye in the retrobulbar area of each animal from both batches. On the 15th day, the solution containing 1 ml of quantum dots (Qdots) labeled mesenchymal adipose tissue stem cells (MSC) was applied in the retrobulbar region of both eyes of batch A rabbits. Batch B animals received 1 ml of PBS solution in both eyes, also in the retrobulbar region.

After 15 days of retrobulbar application of MSC or PBS, the animals were then sacrificed and each optic nerve was fixed for laboratory analysis. Slides were analyzed without prior knowledge of the surgical procedure nor from which batch of animals the study material came.

RETROBULBAR INJECTION

A topical complementary anesthesia with 1% tetracaine hydrochloride eye drops (Allergan) was applied after sedation of the animals, 1 drop in each eye. Pupils were dilated with 1 drop of 1% cyclopentolate eye drops (Allergan) in each eye. After blepharostat placement, a 2mm Wescott scissors incision was made into the upper conjunctiva at 3mm from the limbus to allow access to the retrobulbar region. A blunt tip cannula was inserted through the incision into the Tenonian space. The scleral identification performed with the tip of the cannula was monitored ophthalmoscopically with an indirect ophthalmoscope until reaching the papillary edge of the optic disc. The recommended solution (on the 1st day 1 ml of SF or alcohol, and on the 15th day solution containing stem cells or PBS) was then injected in the retrobulbar space.

CLINICAL EVALUATION OF THE ANIMALS

The eyes of the animals were clinically evaluated using a slit lamp (haag straigt) and light stimulation with a flashlight directed directly to each pupil to analyze the pupillary motility before the retrobulbar procedures, and on the 1st, 7th and 15th days after retrobulbar application in each animal's eye. Conjunctival hyperemia was classified as mild, moderate or severe, presence or absence of ocular secretion, and pupillary reflex.

ANIMAL SACRIFICE

Fifteen days after retrobulbar application of MSC or PBS and immediately after the macroscopic control examination, the animals received a lethal dose of 3% thiopental (25 mg/kg of bodyweight) injected into the marginal ear vein. Immediately after animal sacrifice, the tissues were resected from an ocular globe enucleation. The optic nerve was excised with scalpel blade No. 23 and preserved in 10% buffered formalin. All vials were identified for later inclusion of the paraffin material.

SLIDE PROCESSING

Tissues fixed in 10% buffered formalin were processed in paraffin and filed at the Hematology Laboratory and the Department of Pathological Anatomy of the School of of Medical Sciences. Paraffin tissue blocks were made and cut cross-sectionally with thickness of 12 µm. The slides prepared were analyzed and photographed according to the processing described below:

IMMUNOHISTOCHEMICAL METHOD

The optic nerves preserved in 10% buffered formalin were transferred to a tube with a solution of 5 ml of distilled water and 10% sucrose, and maintained for 24 hours until tissue saturation. The optic nerves were then immersed in a 20% sucrose solution for a further 24 hours, and then fixed in paraffin.

SLIDE CONFECTIONS

The tissue were cut cross-sectionally to the optic nerve following the thickness of 12 µm. The cryostat apparatus of the Department of Pathological Anatomy of the School of Medical Sciences was used. The cuts were applied to the previously silanized slide (approximately 5 cuts per slide) until the tissue was completely extinct. The slides were then analyzed and photographed using a 10X magnification confocal microscope, 854.9nm gain, 488 laser with emission band range from 600nm to 700nm.

Discussion

In Brazil, the occurrence of blindness has been estimated at 0.4 to 0.5% of the population, that is, 4 to 5 thousand people per million inhabitants. Considering that in 2000 the Brazilian population was 160 million inhabitants, the number of blind individuals in the coming years will be much higher than the 640,000 estimated that year.[2,4,30,31]

Although over the last decade research with MSC has brought significant progress applicable to cell therapy, there is still no consensus on some aspects such as the ideal cell marker, the route of administration, the preference for a particular cell type, among others.

The present study sought to monitor the behavior of MSCs originating from the adipose tissue that were applied in the retrobulbar space in the follow-up phase after optic nerve injury. Detection of the presence and permanence of MSCs in the injured tissue became possible due to the pre-labeling of cells with Qdots. Qdots are fluorescent, semiconductor nanoparticles most recently adopted for obtaining bioimages in experimental in vitro and in vivo studies. Among other properties such as photostability and luminescence, they present good resistance to chemical or metabolic degradation, and minimal cytotoxic effects.

Cytotoxic effects are dose dependent, and can be alleviated with the use of low dosages of these nanoparticles.[32] Muller-Borer et al. [32] who used a coculture model during the analysis of confocal images, observed that the number of Qdot-labeled cells did not change substantially 72 hours after labeling. They therefore recommended that in both in vitro and in vivostudies transplantation of Qdot-labeled MSCs should be performed within 24 hours of labeling. Currently, other methods for stem cell monitoring are being studied. Thus, MSCs can be easily monitored after graft and differentiation into host cells. [33,34]

In the present study, Qdots labeling confirmed the presence and distribution of MSCs around the optic nerve in the slides obtained 15 days after application. The 15-day period for MSC monitoring was adopted as a function of Harting et al.[14, 35] experiments which evaluated the behavior of MSC and progenitor cells used for the treatment of traumatic brain injury in rats. In reports by Harting et al. [14] who used the intravenous route for the application of MSCs, approximately 50% of cells that were detected in the brains of rats were present in the injured area or in the penumbra area, i.e., near the injured area. These cells represented a small part of those initially applied. The authors reported that about 48 hours after MSC infusion most cells remained in the lung. About 1.5% to 3.7% of the infused cells were able to cross the lung and reach the arterial circulation. Only about 0.295% reached the carotid artery, and a very small amount (0.0005%) reached and remained in the brain parenchyma. They also reported that in the evaluation made two weeks after the application the number of cells still remaining in the animals' brain was very small, lower than in the initial evaluation.

The intravenous route for MSC transplantation presents the major drawback of significant reduction in the population of transplanted MSC that reach their place of action due to the retention of cells throughout the systemic circulation, being the lung the main barrier. [36]

The search for other more efficient routes of administration that may do without systemic circulation is justified. Harting et al.[35] applied MSCs directly to brain tissue, and reported that in the evaluation carried out 2 days after application almost all transplanted cells were located around the injection site. However, in the analysis carried out after 2 weeks there was already dispersion of transplanted cells.

In the present study, the retrobulbar approach was chosen, and the analysis of the slides corresponding to the 15 days after the application of the MSC showed that there was a significant amount of cells distributed in the penumbra area, i.e., around the injured area of the optic nerve and in its interior, a behavior similar to that reported by Harting et al.[35]regarding the distribution of MSC in the brain tissue.

It is important to emphasize that there was a significant amount of MSC inside the optic nerve (figure 4)A probable explanation is that application in retrobulbar space enables almost all cells applied to reach their place of action.

The presence of MSCs in a significant amount in the most central areas of the optic nerve also suggests that when they are deposited closely on the perineural vascularized areas these cells access more easily the innermost portion of the optic nerve (central retinal artery), which represents a second advantage of the retrobulbar space application technique.

At the 15-day evaluation, the presence of MSC in the eyes of group A animals receiving saline solution was noticeable (figure 4). This finding can be due to the possible local aggression caused by the application of the physiological solution in the retrobulbar space. The permanence of MSCs around the application site may mean that the inflammatory response to the aggression directed the migration and permanence of these cells in the injured area. This behavior was also observed in the eyes of the animals receiving absolute alcohol,and suggests that this is the route of drainage and natural defense of the optic nerve (Figure 4).

The choice of adipose tissue as a source of MSC was guided by the ease of obtaining significant amounts in view of the frequency with which liposuction procedures are performed in our country. The resultads of Manzini et al. [37] and other authors who found adipose tissue to be an excellent source of MSC were also considered.[16-19,37] When subjected to the digestion process, liposuction tissue results in a vascular fraction containing a heterogeneous population of blood-derived cells (granulocytes, monocytes, lymphocytes, and hemopoietic cells), adipose cell stroma, progenitor endothelial cells, pericyte progenitor cells, pericytes, and fibroblasts, among others.[37]

Manzini et al.[37] compared the ability and efficacy of MSC obtained from adipose tissue, umbilical cord, and bone marrow for differentiation into hepatocyte-like cells, and also for the ability to regenerate the liver parenchyma when transplanted as undifferentiated cells. The authors concluded that adipose tissue is an excellent source of MSC, and that when it is obtained from adipose tissue it can be considered as the cell of choice for regenerative therapy of liver tissue. In the present study, the same source and the preparation method for obtaining the SCM were adopted.

The proliferation capacity of MSCs, in addition to immunomodulatory properties and plasticity, their ability to differentiate in the mesodermal lineage, as well as in other cells such as myoblasts, cardiomyocytes, neuronal cells and hepatocytes, suggest to be a promise for regenerative medicine. Due to their biocompatibility, they can also be applied during cell growth in culture media.[32,38]

A protocol for the generation of a functional and transplantable corneal epithelium derived from human induced pluripotent stem cells (iPS) has been initiated in Japan, and the first corneal transplant from pluripotent stem cells has already been successfully carried out.[39]

The possibility of neuronal cell regeneration brings to Ophthalmology the hope of reducing the low vision rates in the world population, a very important aspect since blindness is a limiting factor for every human being. [30,31]

Results

The results of phenotypic, chromosomal stability, and morphological analysis of the stem cells are described below in figures 2 and 3.

Eye images from 12 animals (24 eyes) acquired with confocal optic microscopy confirm the presence of stem cells in 100% of the eyes of animals in batch A receiving Qdots-labeled mesenchymal stem cells, and most intensely in all eyes suffering previous optic neuropathy with absolute alcohol (Figure 4). Images of all negative controls (batch B) are shown in figure 5.

Clinical evaluation of the animals: after the optic nerve injury with absolute alcohol, all rabbits had mild conjunctival hyperemia, yellowish discharge in small amount. During light stimulation, paralytic mydriasis was observed in all left eyes of batches A and B. Rabbit number 12 (batch A) had difficulty walking and balance instability. Ocular hyperemia observed in the eyes of the animals progressively disappeared during the 15 days after the application of absolute alcohol. After the infusion of MSC, all rabbits had a mild reversal of symptoms of hyperemia, improved physical motility, and presented photophobia and myosis to light stimulation in the eyes that previously had paralytic mydriasis. Figure 6 demonstrates the light sensitivity search mode and miosis in one eye of rabbit 11. This method was used in all rabbit eyes as described in the methodology of the present study.

Conclusion

The finding of migration of Qdot-labeled MSCs to the innermost portions of the optic nerve suggests the preservation of their vitality.

The retrobulbar application mode allowed significant amounts of MSC to act locally in the injured areas.

The proximity of the MSC with vascularized areas in the peribulbar region allowed their access to the innermost portion of the optic nerve (central retinal artery), which is the probable access route of the MSC to all suffering areas.

The ease in obtaining stem cells from adipose tissue stimulates the need for studies to define the effectiveness of methods to preserve these cells and their performance in injured tissues.

The permanence of MSC 15 days after retrobulbar application in the analyzed area suggests a possible indicator of cellular vitality and regenerative activity of the neural tissue.

The different methods described in the literature to monitor transplanted MSCs considerably increase the ability to understand the mechanisms to control the death of MSCs, to identify trophic factors and routes of application to improve their grafting.

Acknowlwdgement

To the Experimental Surgery and Medicine Center of the School of Medical Sciences at Universidade Estadual de Campinas (UNICAMP), where the animal experiments were carried out.

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To the Cell Biology Laboratory of the blood center at UNICAMP, where the extraction, differentiation, culture and expansion, and immunophenotypic analysis of mesenchymal cells were carried out.

To Instituto Nacional de Fotônica Aplicada à Biologia Celular -INFABIC where the immunofluorescence slides were analised and photographed.

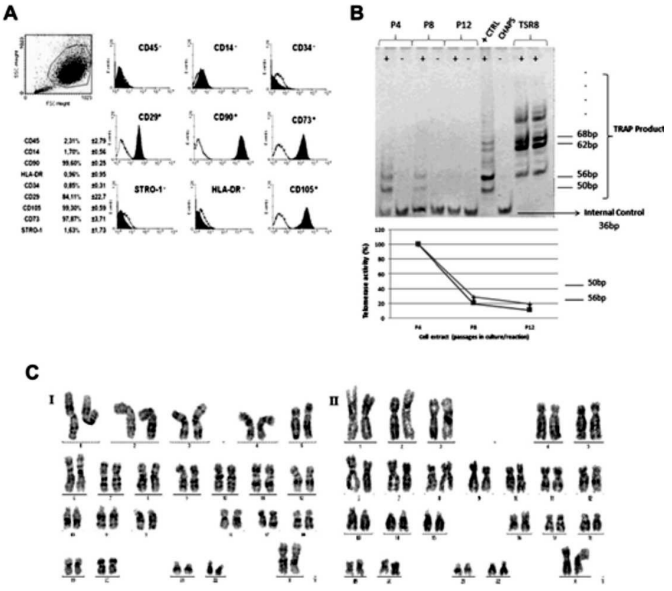


Figure 2: Characterization of MSCs and stability analysis of genetic results. **(A)** Flow cytometric analysis showed that 96.95% of the cells were positive for CD90, CD105, CD73 and CD29 at the 4th pass. The remaining markers CD45, HLA, DR, CD34, CD14 to STRO-1 were less than 1% **(B)** Telomerase enzyme activity was high in the 4th pass, and decreased in the nest passes demonstrating a low instability capacity. **(C)** (I) MSC karyotype analysis in the 8th pass; (II) MSC karyotype analysis in the 10th pass. Cytogenetic analysis showed no abnormality, showing genetic stability.

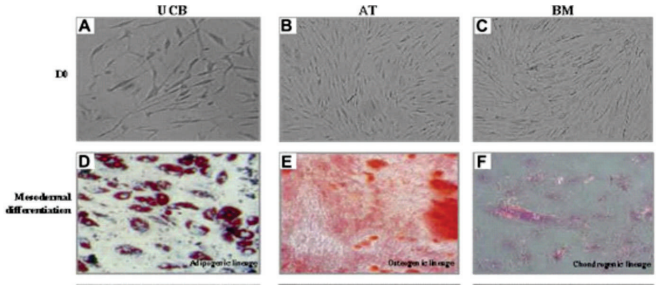


Figure 3: Differentiation of MSCs, mesodermal lineage, hepatocyte differentiation and DHLC functional analysis, glycogen storage, ICG absorption: (A) UCB, (B) AT and (C) BM and MSC undifferentiated (U-MSCs) show fibroblast morphology. AT U-MSC differentiation images in mesodermal lineage exemplifying the 3 different sources of differentiation. (D) AT U-MSCs are differentiated into adipogenic lineage, confirmed by the presence of red oil stained fat droplets. (E) AT U_MSCs undergo osteogenic differentiation confirmed by mineralization, calcium storage, stained with red alizarin. (F) AT U-MSCs undergo chondrogenic differentiation confirmed by the presence of red sirius, resorcin and fuchsin stained chondrocytes.

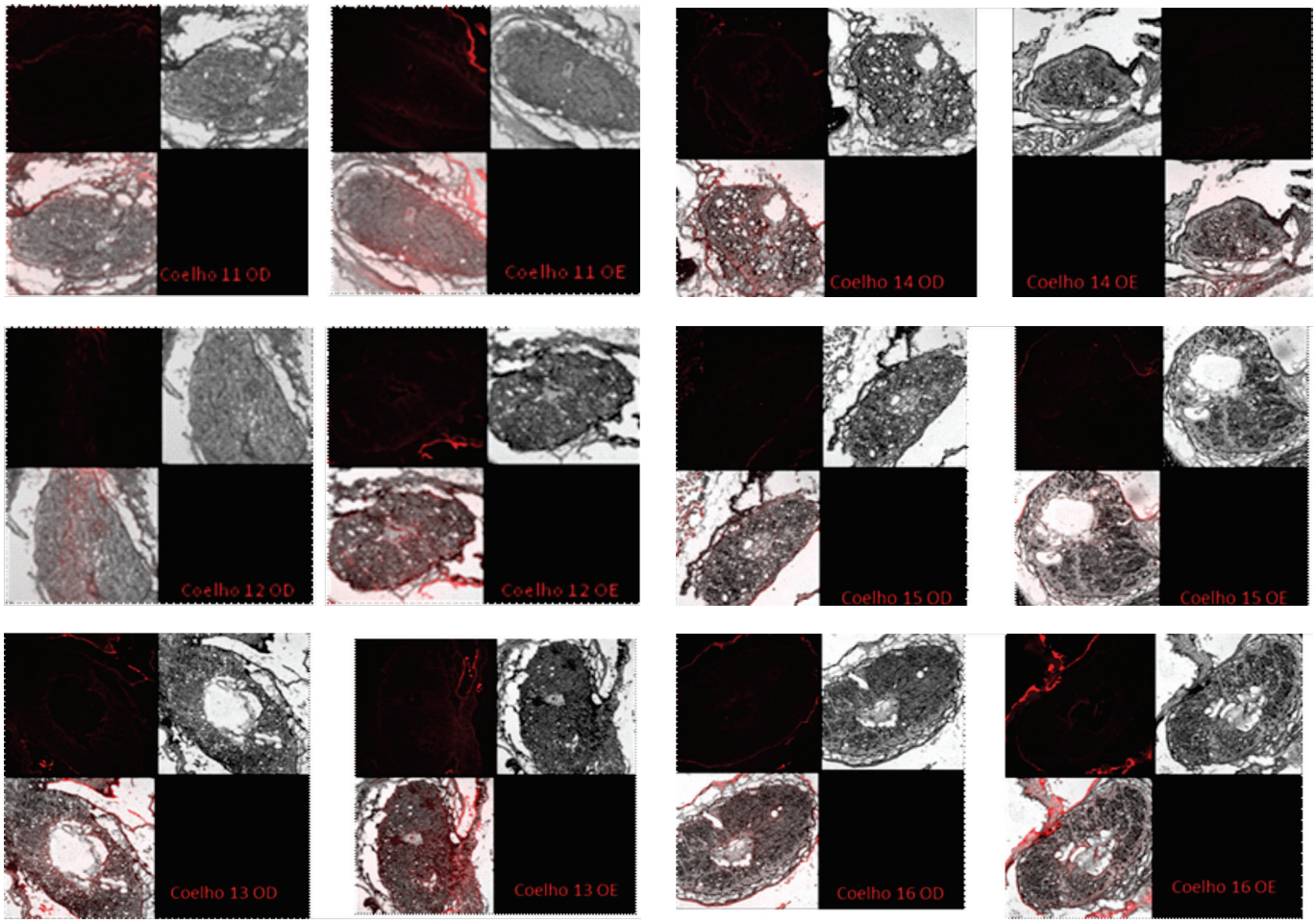
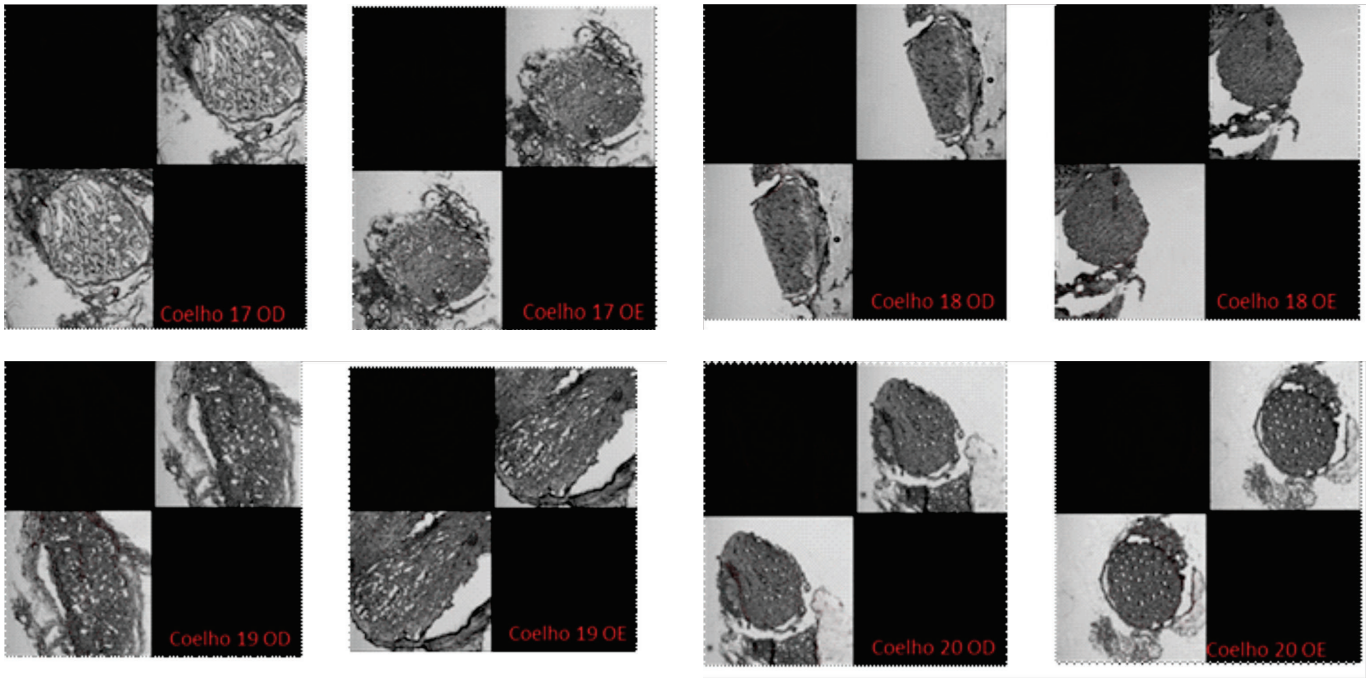


Figure 4: Confocal microscopy : optic nerve of batch A. Rabbits receiving stem cells with Qdots. The upper left image shows the presence of stem cells recorded in red; The upper right image corresponds only to the optic nerve, and the lower left image corresponds to the overlap of the two upper images.



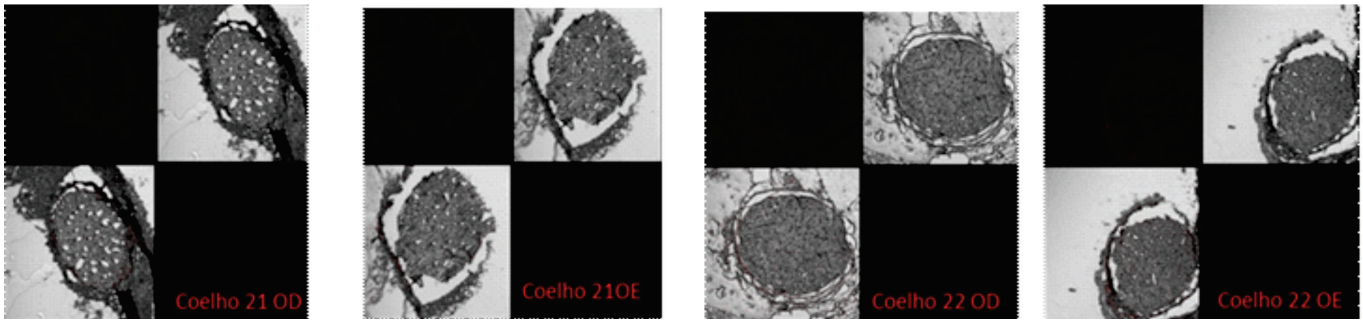


Figure 5: : Confocal microscopy: optic nerve of rabbit from batch B receiving PBS. The upper left image shows the presence of PBS recorded in the immunofluorescence; The upper right image corresponds only to the optic nerve, and the lower left image corresponds to the overlap of the two upper images.

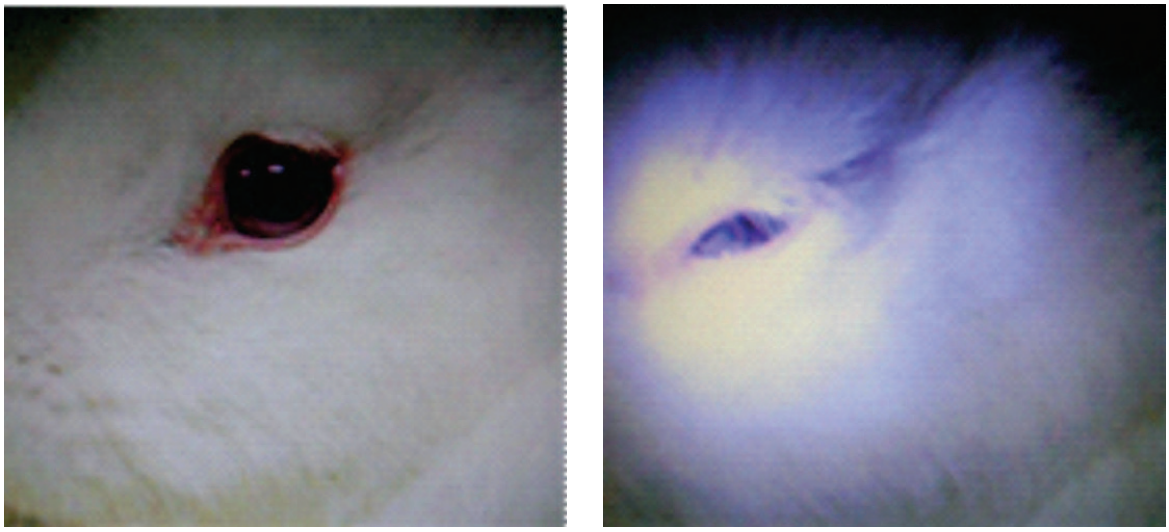


Figure 6: Analysis of pupillary reflex to light. Upper image: LE of Rabbit 11 on the 15th day after the application of MSC before the light stimulus. Below, the same eye during the light stimulus presenting miosis and photophobia.

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