Evaluation of the retina and optic nerve after the use of stem cells for neurodegenerative disorders in an animal model

Estudio de la retina y el nervio óptico tras el uso de células madre en enfermedades neurodegenerativas en un modelo animal

Romana García-Gil^{1*}, Anselmo Feliciano-Sanchez¹, Laura Cubas-Núñez², Jessica Castillo-Villalba², Jorge Fuentes-Maestre³, Mariana Fil³, Jose M. García-Verdugo^{3,4} and Sara Gil-Perotin^{5*}

¹Ophthalmology department, Medical and Surgical Retina Unit, Hospital Universitario y Politécnico La Fe; ²Neuroimmunology and Neuronal Regeneration, Instituto de Investigación Sanitaria La Fe; ³Department of Cell Biology and Parasitology, Universidad de Valencia; ⁴Department of Comparative Neurobiology, Universidad de Valencia; ⁵Department of Neurology, Mixed Unit of Multiple Sclerosis and Neuroregeneration, Hospital Universitario y Politécnico La Fe. Valencia, Spain

Abstract

Objective: To study the neuroprotective and neuroregenerative response of adult mesenchymal cells (aMSCs) on retinal ganglion cells (RGC) and optic nerve (ON) in an animal model of multiple sclerosis (MS) like experimental autoimmune encephalitis (EAE). **Methods:** We studied the changes that occurred in the retina and ON, analyzing the effect of an intravenous injection of aMSCs. Three groups were studied: healthy (control), sick (EAE) and sick treated with aMSCs (EAE-MSC). The animals were monitored using motor disability scales. The retinas and ONs were studied with optical microscopy, immunofluorescence and electron microscopy. **Results:** The results showed that after administration of intravenous aMSCs there was a lower loss of RGC, the average of RGC in the EAE group was of 0.0891 μm compared to 0.166 μm in the EAE-MSC group, with a statistically significant p value ($p = 0.01$). There was a reduction in the inflammatory cell response of the ON (799 cells/ μm^2 vs. 3.69 cells/ μm^2 , $p < 0.0001$), a decrease of myelin loss (overall axonal damage was of 54% compared to 88%) and less axonal destruction (0.16745 axons/ μm^2 vs. 0.3598 axons/ μm^2 ($p = 0.0251$)). **Conclusions:** In this study, we found that after the administration of aMSCs there was a lower loss of RGC, a decrease of myelin loss and a lower degree of inflammation in the ON. However, it would be advisable to expand the methodology to ensure an immunosuppressive and neuroprotective effect in this model.

Key words: Optic neuritis. Experimental autoimmune encephalitis. Mesenchymal cells. Neuroprotection. Immunomodulation.

Resumen

Objetivo: Estudiar la respuesta neuroprotectora y neuroregeneradora de las células mesenquimales adultas (aMSC) sobre las células ganglionares de la retina (CGR) y el nervio óptico (NO) en un modelo animal de esclerosis múltiple, como la

Correspondence:

*Romana García-Gil
Luis Bolinches, 27-10.^a C
C.P. 46023, Valencia, Spain
E-mail: Romygg9@hotmail.com
*Sara Gil-Perotin
E-mail: sara.garcia@uv.es

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encefalitis autoinmune experimental (EAE). **Métodos:** Se estudiaron los cambios que ocurrieron en la retina y el NO, se analizó el efecto de la inyección endovenosa de células mesenquimales. Se estudiaron tres grupos: sanos (control), enfermos (EAE), enfermos en tratamiento con aMSC (EAE-MSC). Los animales fueron monitorizados y seguidos mediante escalas de discapacidad motora. Las retinas y nervios ópticos se procesaron para: microscopía óptica, inmunofluorescencia y microscopía electrónica. **Resultados:** Los resultados demostraron que tras la administración de aMSC intravenosas se producía una menor pérdida de CGR, el promedio de CGR en el grupo EAE fue de $0.0891 \mu\text{m}$, frente $0.1166 \mu\text{m}$ del grupo EAE-MSC, con un valor de p estadísticamente significativo ($p = 0.01$). Se producía una reducción de la respuesta celular inflamatoria en el NO ($7.99 \text{ células}/\mu\text{m}^2$ frente $3.69 \text{ células}/\mu\text{m}^2$; $p < 0.0001$), disminución de la pérdida de mielina (el daño axonal global se reduciría al 54% frente al 88%) y menor destrucción axonal ($0.16745 \text{ axones}/\mu\text{m}^2$ frente $0.3598 \text{ axones}/\mu\text{m}^2$; $p = 0.0251$). **Conclusiones:** En este trabajo, encontramos tras la administración de aMSC, una menor pérdida de CGR, disminución de la pérdida de mielina y menor grado de inflamación en el NO; sin embargo, sería aconsejable ampliar la metodología para poder asegurar un efecto inmunosupresor y neuroprotector en este modelo.

Palabras clave: Neuritis óptica. Encefalitis autoinmune experimental. Células mesenquimales. Neuroprotección. Inmunomodulación.

Introduction

Multiple sclerosis (MS) is considered one of the most common causes of neurological disability in young adults. It is a chronic and inflammatory disease of the central nervous system (CNS), characterized by axonal demyelination, gliosis and neuronal degeneration. A pathogenic hypothesis relates MS with dysimmune responses, with the participation of innate autoantibodies, complement and mediators of immune response¹. Immunosuppressive drugs are considered disease-modifying therapies, as they can reduce disability, relapse frequency, severity and duration of symptoms.

Unfortunately, disease-modifying therapies are not curative and sometimes do not prevent progressive courses or progression of disability².

Cell therapies represent a new frontier in the treatment of neurological diseases. In this context, adult mesenchymal stem cells (aMSC) have demonstrated their therapeutic potential in various disease models, since aMSCs behave heterogeneously according to the tissue of origin and the culture conditions. The International Society for Cell Therapy in 2006, established guidelines for their characterization according to three criteria³: 1) growth adhering to the plastic surfaces used for culture; 2) expression of surface molecules (CD73, CD90 and CD105), and the absence of monocyte and macrophage markers (CD11b and CD14), leukocyte marker (CD45), B cell marker (CD19 or CD79a), hematopoietic parent marker and endothelial cell markers (CD34), and HLA-DR; and 3) in vitro differentiation potential towards osteoblasts, adipocytes and chondroblasts.

The benefit exerted by aMSCs has been associated with the ability to differentiate into several cell lineages, segregate soluble factors crucial to cell proliferation and survival⁴, modulate the immune response⁵ and

migrate to the site of injury⁶. Since tissue damage due to inflammation is a key process triggered in response to injury and disease, aMSCs could become a treatment for any tissue or organic damage associated with an intense inflammatory activity⁵.

Multiple models of MS have been designed in experimental research to achieve a better understanding of the pathogenesis of the disease and the mechanisms of progression. Encephalomyelitis (experimental autoimmune encephalitis, EAE) is an animal model of MS based on active immunization that clinically reproduces the inflammatory lesions that occur in MS. Optic neuritis, an inflammation of the optic nerve (ON) and progressive spinal cord injury have been described in B6/C57 mice after immunization with a fragment of the myelinic oligodendrocyte glycoprotein (MOG) comprising 35 to 55 amino acid residues (MOG₃₅₋₅₅). This induces demyelination with posterior axonal damage and neuronal loss that also affects the eye, particularly retinal ganglion cells (RGC)⁷. In fact, it has been shown that the severity of EAE correlates with the signs of demyelination and inflammation of the ON⁸.

Objective

To study the neuroprotective and neuro-regenerative response of aMSCs on RGC and the ON in an animal model of MS.

Material and Methods

Animals

Twenty-four female B6/C57 mice, 10 weeks old. The animals were divided into three groups: a control group (10 animals), an EAE group (7 animals) and an

EAE-MSC group (animals with EAE treated with aMSCs, administered intravenously; 7 animals). The possible effects of aMSCs injection in control animals were ruled out during the development and optimization process of the animal model. These animals were used for experimentation according to the ethical guidelines for animal experimentation. Animals with EAE were injected with MOG₃₅₋₅₅ (200 µg of peptide/animal, 200 µl intraperitoneally)⁹, monitored every 24-48 h and had a motor disability evaluation performed. The EAE-MSC group was injected with 1×10^6 aMSCs¹⁰ together with a green fluorescent protein (GFP) intravenously into the tail vein. We considered the onset of the disease as the appearance of weakness of the distal tail and/or the presence of a slight irregular movement. When the animals met the endpoint criteria (when the animal was unable to get food or water), they were euthanized. Subsequently, they were perfused through the left ventricle of the heart with 50 ml of sterile saline solution (0.9% NaCl), followed by 150 ml of 4% paraformaldehyde (PFA) or 2% glutaraldehyde, 2.5% PFA solution. Later, the eyeballs and the ON were removed and subsequently fixed in the same fixative for 24 h. Then, they were washed with 0.1 M phosphate buffered saline (PBS) 0.1 M. Finally, the samples were kept at 4 °C in 1.1 M PBS with 0.05% sodium azide to avoid bacterial and fungal contamination.

Growth, characterization and transfection of adult mesenchymal stem cells with fluorescent green protein

All the samples were collected after plastic surgery of patients that underwent elective lipoaspirate procedures at the Cervera Sebastián Clinic (Valencia, Spain). An informed consent form was given to the patients to be signed and stored in the CELULIFE database, after receiving information about the study. The use of adipose tissue samples was approved by the corresponding Ethics Committee (CAEC, Autonomous Committee of Clinical Trials, Valencia, Spain). The participants did not receive a financial compensation. At all times, the study followed the laws and regulations in force, including the ethical principles derived from the Declaration of Helsinki, the international policy on conducting clinical studies in the International Guidelines for Ethical Review of Epidemiological Studies (Council for the International Organizations of Medical Sciences-CIOMS-Geneva, 2009) and Recommendations on Good Clinical Practice.

Isolation of stem cells derived from adipose tissue was carried out using a mechanical and enzymatic method. The digestion mixture was composed of

Dulbecco's Phosphate-Buffered Saline (DPBS) pH 7.5 (Life technologies Cat No.10010056) with 1 mg/mL collagenase type I (Gibco Cat No. 17100-017) and the same volume of aspirated adipose tissue. The tissue was digested for 30 minutes at 37 °C under stirring and then filtered to separate undigested tissue and centrifuged at 500 g for 10 minutes at room temperature. The supernatant (floating adipocytes) was separated from the sediment (fraction of stem cells derived from adipose tissue). The cells were resuspended in an erythrocyte lysis buffer (BD Cat # 555900), incubated at room temperature for 10 minutes and centrifuged at 500 g for 10 minutes at 20 °C. Finally, the cells were resuspended and expanded in Dulbecco's Modified Eagle Medium (DMEM) culture medium with low glucose content (Gibco 31885023), supplemented with 10% human serum and 1% (v/v) penicillin/streptomycin (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, Gibco Life Technologies, No. 15140-122). Stem cells derived from adipose tissue were grown at 37 °C in an air atmosphere with 5% CO₂, with medium change every 3 days. When the cells reached approximately 80% confluence in the flask, the subculture (passage) was performed by trypsinization, counted and reseeded in a T75 cell culture plate treated with polylysine for monolayer cell growth at a density of 5,000 cells/cm². For trypsinization, the culture medium was aspirated, and the flask was washed using PBS. Then, the solution was discarded, and trypsin was added (6 mL/flask) and left for 5 minutes. Trypsin was neutralized using the same amount of complete medium. The cell suspension was centrifuged (500 g for 7 minutes) and its supernatant was aspirated. The sediment was resuspended in complete medium and the cells were counted using the trypan blue exclusion test (hemocytometer). In the fourth step, they were transfected by electroporation with a plasmid expressing GFP. The expression vector was constructed by inserting the commercially obtained empty vector (Invitrogen) pCEP4 into the GFP gene. The plasmid was introduced at the appropriate concentration, and 24 hours later it was possible to determine the effectiveness of the process by fluorescence detection. This plate was incubated for 3 days at 37 °C with 5% CO₂. The cells were fully characterized as aMSCs by immunophenotype with flow cytometry¹¹.

Processing of the optic nerve and retina for histological study

Longitudinal sections of the eyeball (400 µm thick) were obtained with a vibratome (Vibratome Leica

Biosystems 1000mM). The ON sections were selected at the point where it exits the eyeball and underwent two different procedures: 1) cryoprotection in 30% sucrose, followed by rapid freezing with liquid nitrogen and 10 μm sectioning with a cryostat (CM1900, Leica, Heidelberg, Germany), followed by staining with hematoxylin-eosin and toluidine blue; 2) inclusion in epoxy resin followed by 1.5 μm -semifine sectioning with an ultramicrotome (Ultracut, Leica ultracut-MZ6). Resident and reactive glia was detected in frozen 10 μm sections by immunoblotting with rabbit polyclonal antibody anti-GFAP (glial fibrillary acidic protein) (# 12692777, Fisher Scientific, Hampton, New Hampshire, USA) diluted 1:200, incubated with diluted blocking buffer (5% normal goat serum (NGS), 5% casein in PBS) overnight at 4 °C. The samples were washed in 0.1M PB, and incubation of the secondary antibody (goat anti-rabbit alexa 555, # A21428, Fisher Scientific) diluted 1:500 in 5% (NGS) PBS was carried out for 1 hour at room temperature, conjugated with fluorescence. The samples were then washed in 0.1M PB and mounted with aqueous medium (Fluorsave®).

QUANTITATIVE ANALYSIS OF HISTOLOGICAL AND IMMUNOHISTOCHEMICAL SAMPLES

In the sections stained with hematoxylin-eosin/toluidine blue, 246 photographs were taken, 3 sections were selected per sample and 3-4 photos were taken per section, depending on the presence of ON, retina, or both. Finally, 80 photographs of the control group, 84 photographs of the EAE group and 82 photographs of the EAE-MSC group were analyzed. RGC numbers per unit length were measured at an average distance of 300 μm on both sides of the ON entrance site, a reference area with less anatomical variability to allow reproducible counts in different samples. RGCs were counted manually. To study endogenous inflammation, resident and reactive glial cells were detected with anti-GFAP antibodies. Total GFAP positive cells per micron were counted in 3 sections/animal per group, and the differences were analyzed.

QUANTITATIVE ANALYSIS BY ELECTRON MICROSCOPY: G-RATIO AND AXONAL DENSITY

For quantitative studies of transmission electron microscopy (TEM), the ONs were cross-sectioned. Sections with both ON and retina were selected, and then ultra-thin sections, of 70-80 nm, were obtained in resin (Leica EM-UC6) to complete the morphological-ultrastructural

analysis by electron microscopy (Transmission electron microscopy FEI-Tecnai-Spirit-G2, Oregon, USA).

The G-ratio was obtained as the ratio between the internal axonal diameter (excluding myelin) and the total outer diameter (including the myelin sheath). In the ultrafine sections of the ON, more than 100 axons per group were studied and classified as normal (G-ratio: 0.740-0.84)¹², remyelinating axons (G-ratio: 0.85-0.94) and axons with severe myelin damage (G-ratio: 0.951). We obtained 103 micrographs by transmission electron microscopy (48 from the control group, 25 from the EAE group and 30 from the EAE-MSC group). Axonal density was calculated as the number of axons per square micrometer in four sections per animal. This measure was obtained as the average of two blinded independent observers (RGG, LCN).

Statistical analysis

Because our sample size excluded parametric testing, we performed the Mann-Whitney U test to study the statistical significance between experimental groups. The differences between groups were considered significant if the p-value was <0.05. For comparisons between more than two groups, we used the Kruskal-Wallis test.

Results

Animals treated with adult mesenchymal stem cells had a longer disease-free period compared to untreated animals

Animals with EAE experienced the first clinical signs from day 7 to day 19 after immunization. These symptoms consisted of motor deficits secondary to spinal cord injury and were progressive, since our model reproduced a chronic course⁹. The onset consisted of a focal deficit with loss of the muscular tone of the tail, followed by an ascending progressive paresis that led to the endpoint criteria at different time points (the animals were euthanized due to ethical reasons). The clinical course was variable, with an average life expectancy of 57 days (range 30-70). Although there was a delay in the onset of the disease in the EAE-MSC group compared to the EAE group (10 vs. 13 days), p values were not statistically significant ($p = 0.07$). At one point (45 days after immunization), the animals in the EAE group reached an average maximum disability of 3.5 points, where it was evident that the disease was established with severe motor involvement and paralysis

Figure 1. Comparative histological and ultrastructural study of the retina. **A-C:** toluidine blue-stained retinal cryostat sections in the control group (**A**), experimental autoimmune encephalitis group (EAE) (**B**) and EAE group treated with adult mesenchymal stem cells (EAE-MSC) (**C**) (10 μm scale). **D-F:** micrographs, ultrafine sections, ultrastructural study of retinal ganglion cells (RGCs) in the control group (**D**), showing large and rounded nucleus, dispersed chromatin, abundant endoplasmic reticulum and characteristic microtubules (box), EAE group (**E**) and EAE-MSC group (**F**). The morphology of RGCs is altered in the treated group with EAE, and the MSC group shows invaginated nuclei (asterisk) and a more disorganized cytoskeleton (dashed box) (2 μm scale). **G-I:** glial fibrillary acidic protein (GFAP) (red) expression in the RGC layer in the control group (**G**), EAE group (**H**) and EAE-MSC group (**I**) (20 μm scale). **J,K:** microglia cells are characterized by an irregular nucleus, with chromatin condensation in the periphery (asterisks), large elongated cisterns (arrow) and secondary lysosomes (arrowhead) (2 μm scale). **L:** quantitative evaluation of RGC per μm in the three groups studied. **M:** quantitative evaluation of the number of glial cells (GFAP+) in the retina per μm .

of all limbs, making difficult for the animal to obtain food and water, while the animals in the group treated with aMSCs had a maximum score of 2.5 points, with a disease manifested as paralysis of two limbs (both hind or one front and one hind). All animals met the endpoint criteria and were euthanized.

Immunization with experimental autoimmune encephalitis led to morphological changes and decreased the number of retinal ganglion cells with a favorable effect after administration of adult mesenchymal stem cells

aMSCs were transfected with a plasmid expressing GFP in order to detect grafted aMSCs by immunofluorescence. We could not detect aMSCs as GFP+ cells in our retinal or ON samples; however, we observed changes in neuronal viability, myelination and local inflammation, as we describe in more detail.

In both EAE and treated groups, the retinal morphology at low magnification did not differ from that of the control mice (Figs. 1 A-C). However, TEM showed that RGCs in the control group were large cells with rounded nuclei and a central nucleus with dispersed chromatin, abundant rough endoplasmic reticulum and microtubules (Fig. 1 D). In contrast, in the EAE and treated groups, the RGCs cytosol was darker, with smaller nuclei and with large, evident invaginations that reached the center of the nucleus (Fig. 1 E) and with sphericity loss due to acquisition of a cornered shape (Fig. 1 F). They showed a disorganized cytoskeleton that suggested damage of RGCs (Figs. 1 E and F). Adjacent glial cells characterized by light cytosol, intermediate filaments and invaginated nuclei, were observed more frequently in the EAE and treated groups compared to the control group. Microglial cells with a dark cytosol and invaginated form, long cisterns and lysosomes were more abundant in the studied regions of immunized mice (Figs. 1 J and K). Both types of cells were also observed by immunohistochemistry against GFAP, a protein of the intermediate filaments present in astrocytes (Figs. 1 L and M).

RGCs were morphologically altered in immunized mice, but to assess whether morphological changes were related to cell loss, we quantified the number of RGCs per unit length in longitudinal retinal sections in all groups. In accordance with our previous data, the number of RGCs in the control group showed an average of 0.1678 RGC/ μm . The EAE group and the aMSC-treated group showed a significant decrease in

the number of RGCs, with values of 0.0891 RGC/ μm ($p = 0.000035$) and 0.116 ($p = 0.0002$) RGC/ μm , respectively, compared to control mice. When the RGC numbers between the EAE and the aMSC-treated group were compared, the differences were also significant ($p = 0.01$) (Fig. 1 L).

Immunohistochemistry against GFAP showed an increase in GFAP+ cells in the EAE group (0.0419 GFAP+ cells/ μm) and in the treated group (0.0322 GFAP+ cells/ μm) compared to the control group (0.0132 GFAP+ cells/ μm) (Fig. 1 M). These results showed a significant increase in GFAP+ cells in immunized animals compared to control mice ($p = 0.038$) and a partial response to aMSC treatment that, however, did not reach statistical significance ($p = 0.068$).

Endogenous inflammation and demyelination of the optic nerve of immunized mice was partially rescued with intravenous administration of adult mesenchymal stem cells

Morphological examination of the longitudinal sections of the ON with histological staining at low magnification, showed that in the EAE group, the nerve had irregular borders with narrow demarcated regions that corresponded to areas of higher cell density (arrows Fig. 2 C), compared to the absence of ON narrowing in the control group (Fig. 2 A).

These data were confirmed by TEM, since the axons in the ON of the immunized mice showed signs of degeneration such as a dark cytosol with an interrupted microtubule network in the axoneme and myelin spheroid bodies, instead of concentric regular myelin sheaths, as observed in control mice (Fig. 2 D); in addition, abundant reactive astrocytes were observed in the intercellular space between damaged myelinated axons (asterisk in 2 D).

aMSC treatment decreased the degenerative signs in myelin and axons, showed less glial cell infiltrates in the damaged ON and resulted in an intermediate state between the control and treated mice. To determine these changes quantitatively, we obtained cross sections of the ON that were stained with toluidine blue to count cells per unit area (μm^2) (Fig. 2). In the control group, the average number of cells per unit area was of 1.86 cells/ μm^2 , while in the EAE group, it was of 7.99 cells/ μm^2 . aMSC treatment resulted in 3.69 cells/ μm^2 . Statistical studies showed a significant difference between the three groups ($p < 0.0001$). Subsequently, the specific differences between the mean

Figure 2. Comparative histological and ultrastructural study of the optic nerve (ON). **A-E:** toluidine blue stained ON cryostat sections of the control group (**A**), experimental autoimmune encephalitis group (EAE) (**C**) and EAE group treated with adult mesenchymal stem cells (EAE-MSC) (**E**); black arrows: focal irregular borders with increased cell density, 200 μm scale. **B-F:** ultrastructural study of ON axons in the control group (**B**), EAE group (**D**) and EAE-MSC group (**F**); asterisk: astrocytes that fill the spaces due to myelin loss, showing their cell bodies and expansions. These reactive astrocytes are large, occasionally multinucleated cells, with abundant intermediate filaments distributed in the cytoplasm. The nuclei are large, slightly irregular, with loose chromatin, scale 5 μm (**B**), scale 20 μm (**D**) and scale 5 μm (**F**). **G:** cell density in the ON per μm^2 . Significant differences between all groups ($p < 0.05$). **H:** axonal density (number of axons per μm^2). Statistically significant differences favoring the EAE-MSC group ($p < 0.05$).

cellularity in both groups (EAE and EAE-MSC) were compared using the Mann Whitney U test, which was also statistically significant ($p = 0.0143$). To calculate axonal density (Fig. 2 H), we counted the number of

axons per unit area. While in the control group we obtained an axonal density of $0.38049 \text{ axons}/\mu\text{m}^2$, in the EAE group, the value obtained was of $0.16745 \text{ axons}/\mu\text{m}^2$, and this decrease was significant

Figure 3. Study of the degree of myelination. G-ratio. EAE-MSC mice show a demyelination that was recovered after treatment with aMSCs. **A-C:** axonal ultrastructure study. **A:** control: axons with homogenous myelination: normal myelination. **B:** EAE: disorganized axons with great demyelination. **C:** EAE-MSC: disorganized remyelinating axons, 5 µm scale.

($p = 0.0003$). The comparison of axonal density between the EAE and the aMSC-treated group (0.3598 axons/ μm^2) was statistically significant, favoring the treated group ($p = 0.0251$). The G-ratio in EAE-MSC mice showed a demyelination that recovered after treatment with aMSCs (Fig. 3). In the control group, 79% of the axons were normally myelinated, 21% of the axons were remyelinated and none of the axons studied were demyelinated. In contrast, in the EAE group, we found 12% of normal axons, 73% of remyelinated axons and 15% of axons with acute myelin damage. EAE-MSC mice exhibited 46% of axons with normal myelin, 47% with remyelination and 7% demyelinated. It should be noted that, if we consider the percentage of axons that had suffered any damage (grouping the remyelination and demyelination processes) with G-values above 0.84, the total of axons altered was of 88% in the EAE group compared to 54% in the EAE-MSC group.

Discussion

In this work we studied the possible effect of aMSCs, evaluated that an intravenous infusion with aMSCs may improve inflammatory, demyelinating and neurodegenerative damage in both the retina and the ON, while, unfortunately, the clinical effect was not sufficient to stop the progression of the disease.

On the other hand, published studies on different models of axonal damage (transient retinal ischemia¹³ or ON axotomy¹⁴) described that the interruption of RGC axons caused retrograde axonal degeneration that induced RGC death¹⁵. In our work we observed a significant loss of RGCs in EAE mice compared to the control group, which could be the result of documented axonal damage of the ON and Wallerian degeneration. Another possibility would be that, as the inflammation of the ON and the structural damage seemed to be

more relevant, the death of RGCs was caused by the local toxic effect of the microglia, which was increased in the EAE and EAE-MS groups, as reported in other models of ocular inflammation¹⁶. The treatment with aMSCs improved the inflammation of the ON, decreased the loss of RGCs, but no “rescue” effect was observed for RGCs; this could be due to a better access of the aMSCs to the ON through the blood brain barrier¹⁷ or a delayed effect of the decrease in inflammation of the ON due to aMSCs in Wallerian degeneration of the retina.

aMSCs represent a population of multipotent stem cells that, when administered intravenously, have the ability to migrate and graft in places where tissue damage and inflammation occur. We describe that aMSCs administered intravenously had the ability to decrease inflammatory infiltrates and reduce myelin loss and axonal damage. This effect was not related to aMSC grafting, because we could not find green fluorescent aMSCs in our samples. This could be due to several causes, including sequestration in other organs (liver, lungs), immune rejection and/or apoptosis once in the CNS. According to published studies, aMSCs administered by the tail vein in mice initially accumulate in the lungs and gradually pass to the liver, spleen, kidney and bone marrow in the first 48 hours^{18,19}. Rochefort et al. found accumulation in the lung of 50-60% of aMSCs one hour after the injection, with a subsequent decrease to approximately 30% three hours after the injection²⁰. The same group injected aMSCs intravenously in humans and also showed a high pulmonary uptake within 30 minutes²¹. Several studies have demonstrated the low immunogenicity of aMSCs since they do not express MHC-II, that prevents the recognition by dendritic cells and T lymphocytes. However, although they are considered cells that do not cause rejection, when they are exposed to an inflammatory milieu characterized by the presence of high levels of interferon- γ ²², they begin to express HLA-DR (MHC-II), and therefore become immunogenic and are susceptible to rejection. However, our studies and others show that, in addition to the low efficiency for CNS grafting, there is a neuroprotective effect after administration of aMSCs. This effect, called “by-stander”, has been described in studies of myocardial infarction after the infusion of aMSCs, where clinical and functional improvement was observed, but it was not possible to identify grafted cells or differentiated myocytes^{23,24}. aMSCs are characterized by a high capacity for paracrine communication based on the release of small vesicles

or exosomes²⁵. Exosomes have been attributed for the effect of aMSCs at distance. It could be that the decrease of the inflammatory response observed in our study may be due to the remote release of exosomes with additional neurotrophic and immunomodulatory action. However, the exact mechanisms are not yet fully understood.

Within the limitations of this study is the reduced sample size and the follow-up of the disease with motor disability scales due to the lack of resources; the follow-up with techniques such as optical coherence tomography²⁶ for visualization of retinal structures and the ON in vivo, would have allowed a better monitoring of the disease. It would be interesting in the future to expand this study by overcoming this limitations.

The most interesting contribution of this work is its ophthalmological approach when studying the death of RGCs, as well as the sophisticated techniques used, like electron microscopy.

Finally, we want to emphasize that after the administration of aMSCs, we found a lower loss of RGCs, a decrease of myelin loss and a lower degree of inflammation of the ON; however, it would be advisable to expand the methodology to ensure an immunosuppressive and neuroprotective effect in this model. Since tissue damage due to inflammation is a key process triggered in response to injury and disease, aMSCs could become a treatment for any tissue or organic damage associated with an intense inflammatory activity.

Conclusions

In this work, we found that after the administration of aMSCs, there is a lower loss of RGCs, a decrease of myelin loss and a lower degree of inflammation of the ON; however, it would be advisable to expand the methodology to ensure an immunosuppressive and neuroprotective effect in this model.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code

of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

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