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Adipose derived mesenchymal stem cells partially rescue mitomycin C treated ARPE19 cells from death in co-culture condition

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Summary. Age-related macular degeneration is a retinal disease with important damage at the RPE layer. This layer is considered a target for therapeutical approaches. Stem cell transplantation is a promising option for retinal diseases. Adipose derived mesenchymal stem cells secret growth factors which might play a significant role in RPE maintenance. This study aimed to evaluate human AD-MSCs ability to rescue mitomycin C treated dying ARPE19 cells in co-culture condition.

ARPE19 cells were treated with MMC (50 μg/ml, 100 μg/ml and 200 μg/ml) for 2 hours to induce cell death. These treated cells were co-cultured with hAD-MSCs in indirect co-culture system for 3 days and 3 weeks. Then the viability, growth and proliferation of these ARPE19 cells were evaluated by a cell viability/cytotoxicity assay kit and Alamar Blue (AB) assay. Untreated ARPE19 cells and human skin fibroblasts (HSF) were used as controls.

MMC blocked ARPE19 cell proliferation significantly in 3 days and cells were almost completely dead after 3 weeks. Cell toxicity of MMC increased significantly with concentration. When these cells were co-cultured with hAD-MSCs, a significant growth difference was observed in treated cells compared to untreated cells. hAD-MSCs rescue capacity was also significantly higher than HSF for treated ARPE19 cells.

This study showed that hAD-MSCs rescued MMC treated ARPE19 cells from death. It probably occurred due to undefined growth factors secreted by hAD-MSCs in the medium, shared by treated ARPE19 cells in co-culture conditions. This study supports further evaluation of the effect of hAD-MSCs subretinal transplantation over the RPE degeneration process in AMD patients.

Key words: Human adipose derived mesenchymal stem cells, Age-related macular degeneration, Cell death, ARPE19 cell, Mitomycin C

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Introduction

Age related macular degeneration (AMD) is the most common cause of irreversible legal blindness in older people over 65 years in the developed countries (Ambati et al., 2003) and about 3.4% of Spanish old age population of 65 years or older is suffering from AMD (Spanish Eyes Epidemiological Study Group, 2011). There are two forms of AMD: dry (also known as atrophic) which is the most frequent and wet (also known as neovascular or exudative) (Gottlieb, 2002). The wet form has some effective treatments based on intraocular injections of anti-VEGF agents, but to date there is no treatment for the dry forms of AMD. One of the main events of dry AMD is the degeneration and disappearance of the retinal pigment epithelium (RPE) layer of the retina. RPE plays a crucial role in maintaining the adequate functions of neuroretina. If the degeneration of this trophic layer, RPE, of the retina could be prevented somehow or at least delayed, AMD could probably be halted. Several growth factors which

play important roles in maintaining the health of the neuroretina as well as the RPE, which include PEDF, BDNF, VEGF and others, have been identified (Kolomeyer et al., 2011).

New advanced treatments like genetic and stem cell therapy are under investigation, out of which mesenchymal stem cell (MSC) therapy represents a promising alternative (Binder et al., 2007). MSCs are present in several tissues which renew the mesenchymal lineages both under normal and pathological conditions. In addition to replacing the dead and damaged cells the MSC may provide a source of trophic and biochemical positive effects due to having the capacity to secret many immunomodulatory and neuroprotective growth factor molecules (Levkovitch-Verbin et al., 2010; Doorn et al., 2012). Several growth factors like CNTF, BDNF, IGF1, NGF and bFGF secreted by MSC (Labouyrie et al., 1999; Lin et al., 2009) might protect neuroretina and RPE from further degeneration.

Therefore, the secretion of those neurotrophic growth factors by adipose derived MSCs (AD-MSCs) could have positive effects over the survival of ageing RPE and hence might help in slowing the retinal degeneration process in AMD patients. The purpose of this work is to investigate the possibility of rescuing mitomycin C triggered ARPE19 cell death by co-culturing them with human AD-MSCs.

Materials and methods

Cell culture

ARPE19 cells, hAD-MSCs and human skin fibroblast (HSF, ATCC name: BJ cell line) were cultured under standard culture conditions of 5% CO_2 at 37°C in the humidified cell culture incubator in their respective standard culture mediums. The mediums were renewed at 2-3 day intervals. The standard culture mediums for ARPE19 cells, hAD-MSCs and HSF were complete DMEM/F12, DMEM low glucose with glutamine (Gibco, Invitrogen, Paisley, UK) and Eagle's Minimum Essential Medium (EMEM, ATCC formulated cat no-30-2003) respectively, each containing 1/100 penicillin-streptomycin solution supplemented with 10% FBS (Gibco, Invitrogen, Paisley, UK). DMEM/F12 medium also contained 1/100 Amphotericin B solution. On obtaining the confluency (>90%) cells were trypsinized with 0.05% trypsin-tetrasodium ethylenediaminetetra-acetate (Trypsin-EDTA, Gibco, Invitrogen, Paisley, UK), washed and re-suspended in phosphate-buffered saline (PBS; Gibco, Invitrogen, Paisley, UK). Cell numbers and viability for seeding the cells for each experiment were determined by standard Trypan Blue exclusion assay. ARPE19 cell line and HSF was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). hAD-MSCs were gifted by Dr. Teresa Nieto from the Ocular Surface group of IOBA after their routine characterization. The hAD-MSCs used in this study fulfil the minimum criteria for multipotent human mesenchymal stromal cells, as defined by the International Society for Cellular Therapy (Horwitz et al., 2005), such as their multipotent differentiation capacity towards osteoblasts, adipocytes and chondrocytes and the flow cytometry analysis of the panel of MSC specific cell marker (CD73, CD90, CD105, CD45, CD34, CD14, CD19, HLA-DR) expressions (Nieto et al., 2009).

Mitomycin C treatment of ARPE19 cells

Mitomycin C (MMC from *Streptomyces caespitosus*) was purchased from Sigma Chemicals, USA. ARPE19 cells were seeded at the density of 60,000 cells/cm2 in the 24 well plate in standard culture conditions, incubated for 24 hours and allowed to adhere to polystyrene surface properly and to adopt proper morphology. ARPE19 cells were then incubated with MMC in three different concentrations, 50 μg/ml, 100 μg/ml and 200 μg/ml dissolved in the growth medium, in four wells for each concentration for 2 hours. After MMC incubation ARPE19 cells were washed with PBS three times to remove any residual MMC over the cell monolayer. These treated ARPE19 cells were used for co-culturing with hAD-MSCs and HSF.

Indirect co-culture of hAD-MSCs with ARPE19 cells

ARPE19 cells and hAD-MSCs were co-cultivated in the same wells in 1:1 ratio for 3 days as well as for 3 weeks, sharing the same growth medium (complete DMEM/F12 medium) but physically separated by transmembrane of BD Falcon™ cell culture inserts. The ARPE19 cells were cultured in the bottom of the 24-well plates (BD Falcon) and the hAD-MSCs were cultured on the transwell inserts (BD Falcon), the tissue culture treated track-etched polyethylene terephthalate (PET) having a membrane of 0.4 μm pore size (pore density of $2.0\pm0.2 \times 10^6/cm^2$). The hAD-MSCs were also co-cultivated with ARPE19 cells treated with different concentrations of MMC. In another set of experiments, treated and untreated ARPE19 cells were co-cultured with HSF.

Cell viability assay by viability/cytotoxicity assay kit for determining live and dead cells

The cell viability/cytotoxicity assay kit for live & dead cells (Biotium Inc., USA) was used to evaluate cell viability after three weeks of co-culture in accordance with the manufacturer's protocol. The kit provides a two-color fluorescent staining of live (green) and dead (red) cells using two probes; Calcein acetoxymethyl ester (calcein AM) stains live cells green while EthD-III (Ethidium homodimer III) stains dead and damaged cells red. After staining, both cells, APRE19 and hAD-MSCs, were visualized using a fluorescence microscope (Leica Microsystems, Mannheim, Germany) and were photographed at random per well.

Alamar Blue (AB) assay for evaluating cell viability and proliferation

After 3 days of co-culture growth conditions, AB assay was used for evaluating cell viability along with cell proliferation activity. Cells were washed with PBS and complete medium with 10% Alamar Blue (AbD Serotec, Oxford, UK) was added to each well and incubated under standard incubation conditions for 5 hours. The fluorescence intensity of AB was measured (Spectra Max M5 Multi-Mode Microplate Reader, Molecular Devices LLC, US) with excitation wavelength at 560 nm and emission wavelength at 590 nm. The degree of AB reduction corresponded to the number of live cells and cell proliferation activity. The negative control for the measurement of fluorescence intensity was taken as the medium with AB reagent without any cells. The viability and proliferation activity of cells in different culture conditions is directly correlated with the degree of reduction of AB, which is reflected by the fluorescence intensity measured for each sample. The experiments were performed three times each with duplicates. The mean value of arbitrary fluorescence intensity unit (AFU) ± standard error of mean (SEM) were plotted against all the different samples of the experiments. The diagrammatic presentation of the experiment is shown in Fig. 1.

Statistical analysis of AB fluorescence readings

All the samples are quadruplicated in the microplate reader to avoid any pipetting errors. All the experiments were repeated three times in duplicates to check the reproducibility of the trends observed. The readings obtained from three times repetition of the experiments were subjected to statistical analysis through MS Excell programme and online GraphPad Software. Standard deviations (SD), standard errors of means (SEM), means and p-values were calculated and the observed statistical significance was adjusted to $P<0.05$ and $P<0.01$. T-test (paired/two tailed) was used to compare the different means of each treatment group obtained by the three experiments.

Results

Mitomycin C treatment triggers ARPE19 cell death

Fluorescence microscopy as well as AB assay analysis results showed that MMC (50 μg/ml, 100 μg/ml and 200 μg/ml) treatment reduced ARPE19 cell proliferation in 3 days significantly ($p<0.05$, n=3) and almost all ARPE19 cells died completely after 3 weeks (Figs. 2, 3). The results obtained after cell viability/ toxicity assay at 3 weeks in Fig. 2 show the pattern of

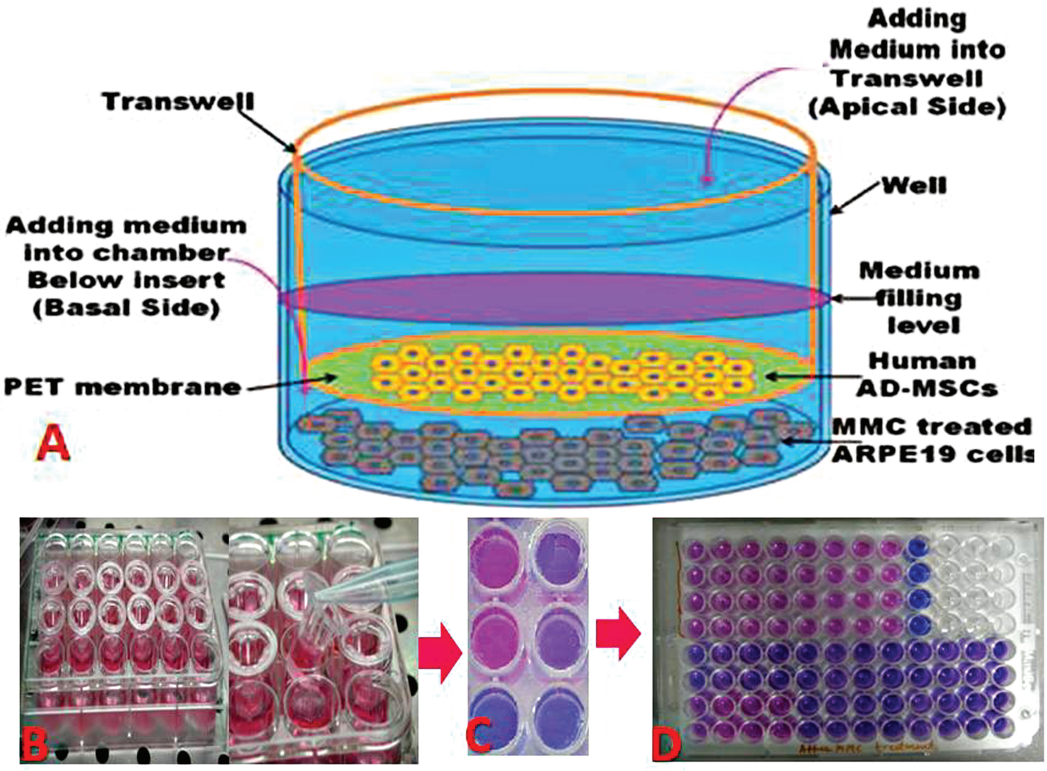

Fig. 1. Diagrammatic representation of the experiments carried out. **A.** Schematic representation of indirect co-culture system **B.** Indirect co-culture system in which MMC treated ARPE19 cells were co-cultivated with AD-MSCs. **C.** Incubation of ARPE19 cells with Alamar Blue **D.** Reading of fluorescence of incubated AB medium in quadruplets using microplate reader.

cell survival and death under different culture conditions by green and red fluorescence, respectively. The ARPE19 cells in control without any treatment showed 100% viability (Fig. 2A) while ARPE19 cells with 50 μg/ml MMC treatment were almost dead (Fig. 2B) after 3 weeks.

When proliferation activity of ARPE19 cells cultured for 3 days was measured by AB assay, a significant high fluorescence intensity ($p<0.05$, n=3) was found in the wells with untreated ARPE19 cells compared to the wells with MMC treated ARPE19 cells (table 1). Fluorescence intensity decreased significantly ($p<0.05$, n=3) with the increase of concentration of MMCs (50 μg/ml, 100 μg/ml and 200 μg/ml) (Fig. 3,

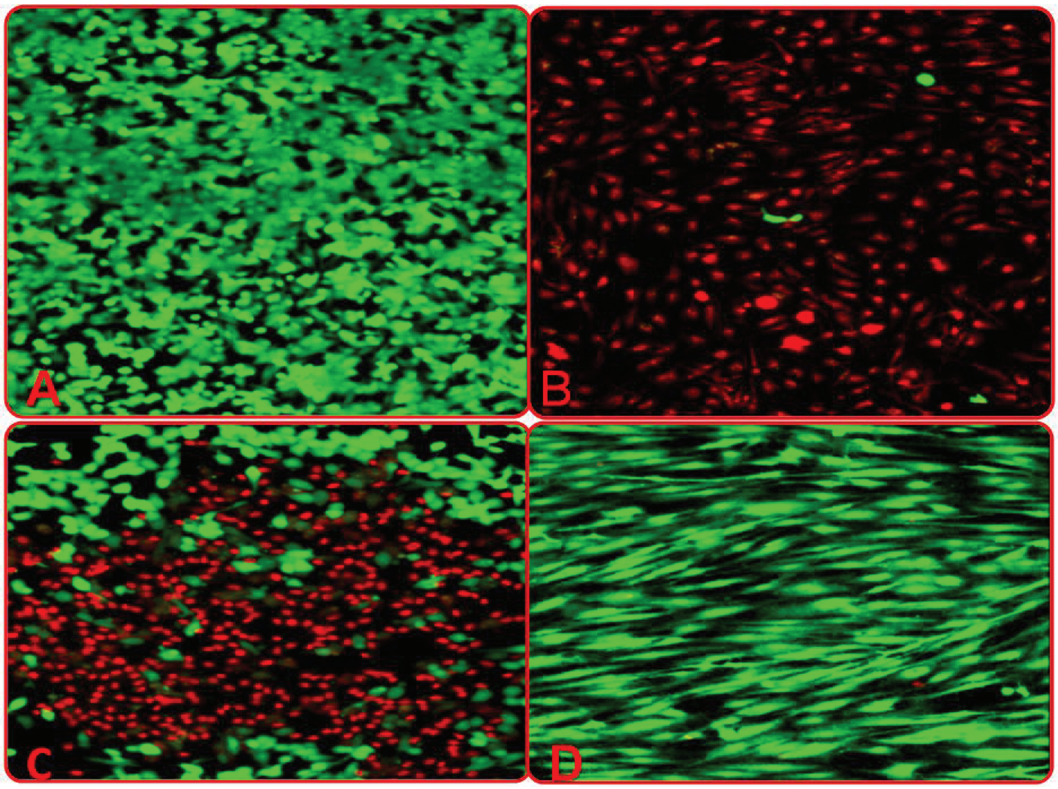

Fig. 2. Viability /Cytotoxicity assay of MMC treated and untreated ARPE19 cells co-cultivated with hAD-MSCs for 3 weeks in standard culture condition. Green fluorescence indicates live cells and red fluorescence indicates dead cells **A.** Untreated ARPE19 cells. **B.** ARPE19 cells treated with 50 μg/ml MMC. **C.** ARPE19 cells treated with MMC and co-cultured with hAD-MSCs. **D.** hAD-MSCs on Falcon transwell.

Table 1. Statistical data analysis of proliferation of MMC treated (50 μg/ml, 100 μg/ml and 200 μg/ml) and untreated ARPE19 cells co-cultivated with hAD-MSCs or HSF for 3 days in standard culture conditions.

Culture conditions	Mean AFU (n=3)	SD	SEM (n=3)	P-value (*)	P-value (**)
ARPE19 cells only	17,928.89	967.40	558.55		
ARPE19 cells+MSCs on TW	18,625.14	885.29	511.14	0.0437	
ARPE19 cells +50 ug MMC	12,720.92	2,884.66	1,665.47		0.0486
ARPE19 cells+50 ugMMC+MSCs on TW	17,106.12	1,803.09	1,041.09	0.0312	
ARPE19 cells+100 ugMMC	10,088.56	2580.21	1,489.68		0.0153
ARPE19 cells+100 ug MMC+MSCs on TW	14,838.51	2304.66	1,330.60	0.0194	
ARPE19 cells+200 ugMMC	5,238.20	647.97	374.11		0.0492
ARPE19 cells+200 ugMMC+MSCs on TW	8,295.78	780.99	450.91	0.0205	
ARPE19 cells+50 ugMMC+HSF on TW	14,326.74	374.51	216.22	0.0467	

Mean AFU reflects the means of AFU values obtained from AB assay. SD and SEM are standard deviation and standard error mean respectively, associated with corresponding mean AFU values. Single asterisk (*) represents the significant P-value between MMC treated or untreated ARPE19 cells vs MMC treated or untreated ARPE19 cells co-cultivated with AD-MSCs or HSF. Double asterisk (**) represents the significant P-value between untreated vs MMC treated ARPE19 cells. Statistical significance is adjusted at $P<0.05$ (*).

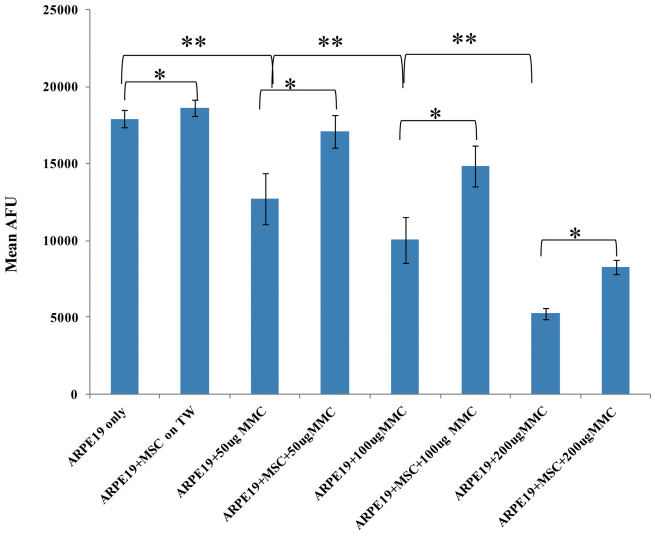

Fig. 3. MMC treated (50 μg/ml, 100 μg/ml and 200 μg/ml) and untreated ARPE19 cells proliferation in co-culture conditions with hAD-MSCs at 3 days. Alamar Blue assay was used to detect the cell number as well as cell proliferation activity and data is presented as mean arbitrary fluorescence unit (AFU) vs cell culture conditions. All values shown are corrected with background fluorescence of the reagent in medium alone. The bar represents mean value of fluorescence intensity (AFU) of three different experiments in duplicated wells ± SEM. Single asterisk (*) and double asterisk (**) represent the significant P-value between different culture conditions as presented in graph. Statistical significance is adjusted at P<0.05.

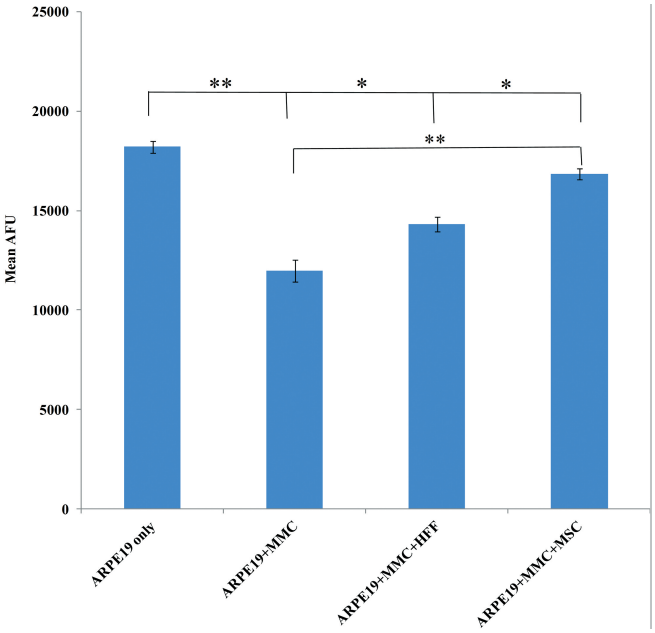

Fig. 4. MMC treated (50 μg/ml) and untreated ARPE19 cells proliferation in co-culture conditions with hAD-MSCs and HSF separately at 3 days. Alamar Blue assay was used to detect the cell numbers as well as cell proliferation activity and data is presented as mean arbitrary fluorescence unit (AFU) vs cell culture conditions. All values shown are corrected with background fluorescence of the reagent in medium alone. The bar represents mean value of fluorescence intensity (AFU) of three different experiments in duplicated wells ±SEM. Single asterisk (*) and (**) represent the significant P-value between deferent culture conditions as presented in graph. Statistical significance is adjusted at P<0.05.

table 1).

MSCs partially rescue the MMC-treated ARPE19 cells from death

Cell viability/toxicity assay demonstrated that 50 μg/ml MMC treated ARPE19 cells after three weeks retained significant viability, when co-cultured with hAD-MSCs (Fig. 2C) and there was no adverse effect after three weeks on the hAD-MSCs grown on transwell due to death of MMC treated ARPE19 cells in the well (Fig. 2D).

AB analysis showed that in each treatment group the ARPE19 cells co-cultured with hAD-MSCs expressed significantly higher fluorescence intensity ($p<0.05$, $n=3$) than ARPE19 cells alone (Fig. 3, Table 1).

As a control, in a further experiment a comparative study using AB assay kit was done to determine the effects of hAD-MSCs and HSF separately on 50 μg/ml MMC treated ARPE19 cells. The results showed that the wells with hAD-MSCs had significantly higher fluorescence intensity ($p<0.05$, $n=3$) than the wells containing HSF (Fig 4).

Discussion

Cell therapy approaches using differentiated human embryonic stem cells (hESCs) are currently under evaluation as a treatment for dry AMD patients (Klimanskaya et al., 2004). A clinical trial is undergoing in this respect by Advanced Cell Technology, USA (ClinicalTrials.gov identifier: NCT01344993). However, apart from ethical issues, it seems to be a complicated procedure due to a number of issues; obtaining hESCs in sufficient quantity, implanting hESCs-RPE cells with a suitable cell carrier in patients, control over the need of using immune-suppression, a possible graft rejection, postsurgical complications for graft integration as well as securing implanted cell survival *in vivo* in sufficient amounts for detaining dry AMD progression in the patients. Even more, the high cost of implementing this procedure in each patient makes it far from the access of millions of people suffering from dry AMD all over the world. Therefore, it would be worth looking for some other procedures that can utilize the protective capacity of patients' own cells for stopping or delaying the progress of RPE cell degeneration at initial stage, thus delaying AMD progression and which can be applicable effectively in all AMD patients.

Published reports showed that sub retinal implanted bone marrow derived MSCs could integrate and

differentiate partially into RPE cells in animal models such as mice (Atmaca-Sonmez et al., 2006). This supports the feasibility of using mesenchymal stem cells as a potential candidate for transplantation in suretinal space. However there are no reports evaluating hAD-MSCs rescue capacity on dying RPE cells as has been achieved in this study.

MSCs can be obtained from different sources and are used in clinics routinely for cell based treatment for replacing dead and damaged cells, such as skin treatment, due to their capacity to differentiate into different lineages (Sasaki et al., 2008). MSCs also secret a number of immunomodulatory and neuroprotective trophic factors that might protect degenerating retina and RPE cells. MSCs derived from patients' own tissues provide autologous nature to the cells and support to overcome the problems generated due to immunoreactivity (Hilfiker et al., 2011). Out of different tissue sources of MSCs, adipose tissue seems to be one of the most appropriate because of easy procedures to obtain it in sufficient amount (De Ugarte et al., 2003).

MMC is a bioreductive DNA alkylating agent (Rockwell et al., 1982) which was used to inhibit cell proliferation and to induce cell death as well. It has been used to induce cell death by apoptosis as well as necrosis in several publications (Hofman et al., 1998; Wu et al., 2002, 2008; Park et al., 2002). A recent paper showed that MMC-induced cellular apoptosis in corneal endothelial cells was mediated through Caspase-8, Caspase-9, and the mitochondrial regulated pathways, as well as through upregulation of p53-dependent and p21-dependent signal transduction pathways (Wu et al., 2008). Oxidative stress over time has been hypothesized to underlie the development of AMD. Oxidative stress directly targets mitochondrial-dependent apoptosis that requires release of cytochrome c from mitochondria and subsequent activation of a specific class of cytoplasmic proteases known as caspases (Takahashi et al., 2004). Thus, it seems that MMC induced apoptosis in ARPE19 cells is quite similar to apoptosis in the degenerating RPE cells of the AMD patients.

AB assay analysis results obtained by treating ARPE19 cells with MMC at different concentrations of 50 μg/ml, 100 μg/ml and 200 μg/ml for 2 hours in standard culture condition followed by growing them for three days showed that MMC was able to partially inhibit ARPE19 cell proliferation (Fig. 3). Calcein AM/EthD-III probes of cell viability/cytotoxicity kit could measure two recognized parameters of cell viability; intracellular esterase activity and plasma membrane integrity, respectively, in ARPE19 cells which were under MMC triggered cell death process. The results obtained using these probes clearly showed that after three weeks cells were completely dead if treated with 50 μg/ml MMC (Fig. 2B). This confirmed the suitability of using this concentration of MMC for further study.

In a further study, the hAD-MSCs were co-cultivated for three days and three weeks with MMC treated and untreated ARPE19 cells using transwells. AB assay at three days demonstrated that there was significant growth difference between ARPE19 cells co-cultured with hAD-MSCs and ARPE19 cells in MMC treated or untreated group. This result confirmed that the hAD-MSCs support the ARPE19 cells proliferation as well as partially rescuing the ARPE19 cells triggered for cell death. The fluorescence microscopic observations using viability/toxicity assay kit at three weeks further confirmed that hAD-MSCs are capable of rescuing partially the dying ARPE19 cells (Fig. 2C). It is hypothesized that the several factors secreted by hAD-MSCs are involved in this partial rescue process. These putative protective factors could be CNTF, BDNF, IGF1, NGF and bFGF etc according to the literature (Labouyrie et al., 1999; Lin et al., 2009). The identification and measurement of these putative protective factors present in supernatants collected in different incubation conditions are still under development. The approach used for this development would be to block reciprocal influences of these putative protective factors on hAD-MSCs and ARPE19 cells. The results also showed that hAD-MSCs maintained their viability for three weeks on track-etched PET transwells. This showed that factors released by dying ARPE19 cells do not affect the viability of hAD-MSCs but their effects on the differentiation towards a cell lineage could not be ruled out and it is still under evaluation.

As evident from the graph in Fig. 3, the proliferation of MMC treated ARPE19 cells decreased as the concentration of MMC increased from 50 μg/ml to 200 μg/ml. Co-culturing of these MMC treated ARPE19 cells with hAD-MSCs showed that treated ARPE19 cells in co-culture condition were able to proliferate significantly higher than those cells that were not in co-culture condition. However, they proliferated less than the ARPE19 cells under untreated conditions. This indicates that the putative factors released in co-culture medium were creating a microenvironment which might be conducive to rescuing the ARPE19 cells from the cell death process. In *in vivo* conditions, in dry AMD patients, RPE cells are under degenerating stage and implanting hAD-MSCs in the subretinal space might create a microenvironment as observed in *in vitro* conditions, to rescue partially the degenerating RPE cells, thus helping to prevent AMD progression.

To confirm further that this rescue capacity is only associated with hAD-MSCs, and that other types of cells such as fibroblasts can have different grades of rescue capacity, we compared the effects of hAD-MSCs and HSF over MMC treated ARPE19 cells in co-culture conditions. The results in Fig. 5 confirmed that hAD-MSCs have a higher capacity to rescue ARPE19 cells than the HSF. However, fibroblasts have different characteristics from hAD-MSCs (Alt et al., 2011) and are involved in producing some retinal diseases, such as proliferative vitroretinopathy (PVR) complications (de Souza et al., 1995), thus it encourages further hAD-MSCs *in vivo* study.

Thus, it is concluded that the hAD-MSCs partially rescue MMC treated dying ARPE19 cells in *in vitro* conditions, probably by creating a microenvironment containing many factors released in co-culture conditions. The results strongly support making a strategy for future hAD-MSC therapy for dry AMD patients.

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Declaration of interest. The authors do not have any conflicts of interest.

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