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Original Research

PEDF Prevents Mitochondrial Function Decay and ER Stress Induced by Rotenone in Aging RPE Cells

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Abstract

Background: Neurodegenerative diseases, including age-related macular degeneration (AMD), may be linked to mitochondrial dysfunction and endoplasmic reticulum (ER) stress. We examined whether Pigment epithelium-derived factor (PEDF) could prevent changes in the structure and function of these organelles by accelerating by rotenone (ROT), a mitochondrial inhibitor, in human retinal pigment epithelium (RPE) cells of chronological age. **Methods**: RPE cells from 9–20, 50–55, 60–70, and >70-year-old donors were isolated, grown as primary cultures, harvested, and treated with ROT and PEDF for electron microscope (EM), western blot analysis, and polymerase chain reaction (PCR). Reactive oxygen species (ROS) and cytoplasmic calcium $[Ca^{2+}]_c$ and mitochondrial calcium $[Ca^{2+}]_m$ levels were measured by flow cytometry using 2′,7′-dichlorodihydrofluorescin diacetate (H2-DCF-DA), fluo-3/AM, and Rhod-2/AM, and ATP levels were measured using a luciferin/luciferase-based assay. Mitochondrial membrane potential ($\Delta\Psi m$) was detected using 5,5′,6,6′-tetrachloro1,1′,3,3′-tetraethylbenzimid azolocarbocyanine iodide (JC-1), and susceptibility of the cells to ROT toxicity and PEDF-protective effect was determined by propidium iodide (PI) staining and lactate dehydrogenase (LDH) assay. The expression of ER stress-related genes was detected using real-time (RT)-PCR. **Results**: We observed decay in the mitochondria of aged RPE cells, including matrix abnormalities, elongation, loss of cristae, and disruption of membrane integrity after ROT treatment. We also observed lower $[Ca^{2+}]_c$, higher ROS and $[Ca^{2+}]_m$ levels, decreased $\Delta\Psi m$ after ROT treatment, and greater susceptibility to ROT toxicity in aged RPE cells. PEDF can protect the cristae and integrity of the mitochondrial membrane, increase ATP levels and $\Delta\Psi m$, and lower ROS, $[Ca^{2+}]_c$, and $[Ca^{2+}]_m$ in aged RPE cells induced by ROT. In addition, there was an increase in RDH expression in RPE cells with increasing age after PEDF treatment. Similarly, PEDF decreased the expression of ROT-induced ER stress-related genes. **Conclusions**: Our study provides evidence that PEDF can reduce bioenergetic deficiencies, mitochondrial decay, and ER stress in aging RPE, a condition that may trigger the onset of retinal diseases such as AMD.

Keywords: PEDF; RPE cells; rotenone; mitochondria; ROS; Ca^{2+}

1. Introduction

Retinal pigment epithelium (RPE) is a monolayer of cuboidal cells located between the retinal neurosensory photoreceptors and choriocapillaris, playing a crucial role in visual processing. Disruptions in these processes and defects in the RPE result in retinal degeneration and contribute to the progression of age-related macular degeneration (AMD) [1–8]. AMD is a multifactorial disease, and its etiology, in part, stems from age-related cumulative oxidative damage to the RPE due to an imbalance between the generation and elimination of reactive oxygen species (ROS) [9–11].

Mitochondria (MT) serve as the main source of cellular ROS and adenosine triphosphate (ATP), and play a vital role in regulating cellular survival and death mechanisms [12–14]. Therefore, mitochondrial health and activity are central to the aging process, with evidence suggesting a link, albeit a tenuous one, between mitochondrial respiration and longevity. Moreover, compelling evidence suggests that age-related mitochondrial dysfunction is an initiating factor in various neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), and AMD [15–19]. The endoplasmic reticulum (ER) is an important organelle involved in the biosynthesis of proteins, lipids, and sugars, as well as cellular homeostasis [20]. Previous studies have revealed that MT and the ER play a key role in regulating neurological activities. Altered ER-mitochondrial signaling, resulting in mitochondrial damage, ER stress, dysregulation of Ca^{2+} homeostasis, lipid metabolism defects, and autophagy, is common in neurodegenerative diseases [21,22]. ER stress mediated by oxidative stress can overactivate autophagy, leading to RPE dysfunction [23,24]. Although AMD primarily involves photoreceptor damage in the central retina, histological changes in the RPE precede vision loss in the early stages of AMD [25]. Oxidative damage-induced mitochondrial dysfunction and ER stress are the key contributors to the pathogenesis of ADM, given the susceptibility of the RPE to oxidative stress [1,26–28].

Pigment epithelium-derived factor (PEDF), a glycoprotein belonging to the superfamily of serine protease inhibitors, is a highly effective inhibitor of angiogenesis in cell culture and animal models [15,29]. In addition, PEDF has been detected in the vitreous humor, retina, and choroid, with decreased levels observed in AMD [16,17]. The therapeutic potential of PEDF has been extensively studied *in vitro*, revealing its antioxidant, anti-inflammatory, and pro-survival effects on various ocular cells, including RPE, photoreceptors, pericytes, and ganglion cells [30–33]. Additionally, PEDF evaluation in clinical trials has demonstrated the effectiveness of intravitreal, subretinal, or periocular injections of an adenoviral vector encoding PEDF in suppressing choroidal neovascularization in AMD [18,19,34–39].

Collectively, these findings suggest a common origin of neurodegenerative diseases associated with advanced aging, involving mitochondrial dysfunction and ER stress. Therefore, agents that improve bioenergetic efficiency play a crucial role in combating aging and age-related diseases [40,41]. Targeted interventions that reduce cyclooxygenase activity, ROS production, or lipofuscin accumulation may delay the detrimental effects of oxidative stress on mitochondrial decay, ER stress, and RPE degeneration, ultimately preventing vision loss. In this study, we investigate whether PEDF can protect the structure and function of MT and the ER from damage mediated by ROT-induced ROS in human RPE cells across different age groups. Our findings provide experimental evidence that supports the clinical potential of PEDF in AMD treatment.

2. Materials and Methods

2.1 Ethics Approval

This study applied the same PRE cell lines as our published studies [42]. The study was carried out in accordance with the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Second Affiliated Hospital of Xi'an Medical University.

2.2 Materials

All the tissue culture reagents were obtained from Gibco (Gaithersburg, MD, USA). 2′,7′-dichlorodihydrofluorescin diacetate (H2-DCF-DA), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimid azolocarbocyanine iodide (JC-1), Mito Tracker Green, and Mito Tracker Red were obtained from Molecular Probes (Interchim, Montlucon, France). Goat anti-human UCP2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ROT and the luciferin/luciferase-based ATP assay kit was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the lactate dehydrogenase (LDH) assay kit was purchased from Roche Pharmaceuticals (Nutley, NJ, USA). Stock solutions of ROT (10 mM) were prepared in dimethyl sulfoxide (DMSO).

2.3 Primary Human RPE Cell Culture

Human RPE cells were cultured as previously described [43] and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin-neomycin. Cultures in the third or fourth passage from four groups of human donors, aged 9–20, 50–55, 60–70, and >70 years were used for the experiments. Five to seven different donor RPE lines were used for each age group. As the study we published earlier [42]. All cell lines were validated by STR profiling and tested negative for mycoplasma. Cells were all cultured in a humidified incubator at 37° and 5% CO_2.

2.4 Treatments with Mitochondrial Complex I Inhibitor and PEDF

For each experiment, either 1.25 or 5 µM ROT was added to the cells [44,45]. For some experiments, 100 µg/mL PEDF was added to the cells 2 days prior to the ROT treatment. To measure ROS, mitochondrial membrane potential ($\Delta\Psi m$), mitochondrial fluorescence, cytosolic calcium concentration ($[Ca^{2+}]_c$), mitochondrial calcium concentration ($[Ca^{2+}]_m$), and LDH release and to assess morphological changes in both untreated cultures and cultures treated with ROT, the cells were treated with 5 µM ROT for 1 h. For experiments involving evaluation of morphological changes in the mitochondria using electron microscopy, measurement of cellular ATP levels, and assessment of cell viability using propidium iodide (PI) staining, the cells were treated with 1.25 µM ROT for 24 h. For RT- and real-time PCR, and western blot analysis, the cells were treated with 100 µg/mL PEDF for 48 h.

2.5 PI Staining and LDH Assay

Cell death in the cultures was evaluated by measuring red fluorescence and LDH activity in the conditioned medium using PI staining and colorimetric assays, respectively. Briefly, PI staining was conducted by seeding untreated and PEDF-treated RPE cells in 6-well plates at a density of 1×10^6 cells/well and incubating them for 24 h. The untreated and pretreated (100 µg/mL PEDF) cells were then exposed to 1.25 µM ROT for 24 h and 48 h, respectively. The cells were harvested and incubated with 4 µg/mL PI (diluted in phosphate buffered saline [PBS]) for 60 min at room temperature. Subsequently, the cells were rinsed twice in PBS and immediately analyzed by flow cytometry at 488 nm excitation. Red fluorescence was measured at 590 nm. The results were expressed in arbitrary units as median fluorescence intensity.

For LDH analysis, the cells were seeded in 96-well plates at a density of 1×10^4 cells/well. The untreated and pretreated cells were exposed to 5 µM ROT in 50 µL of culture medium for 1 h and 48 h, respectively. After treatment, 50 µL of the culture supernatant from each sample was transferred into a fresh 96-well plate and reacted with 50 µL of the reaction mixture at room temperature for 30

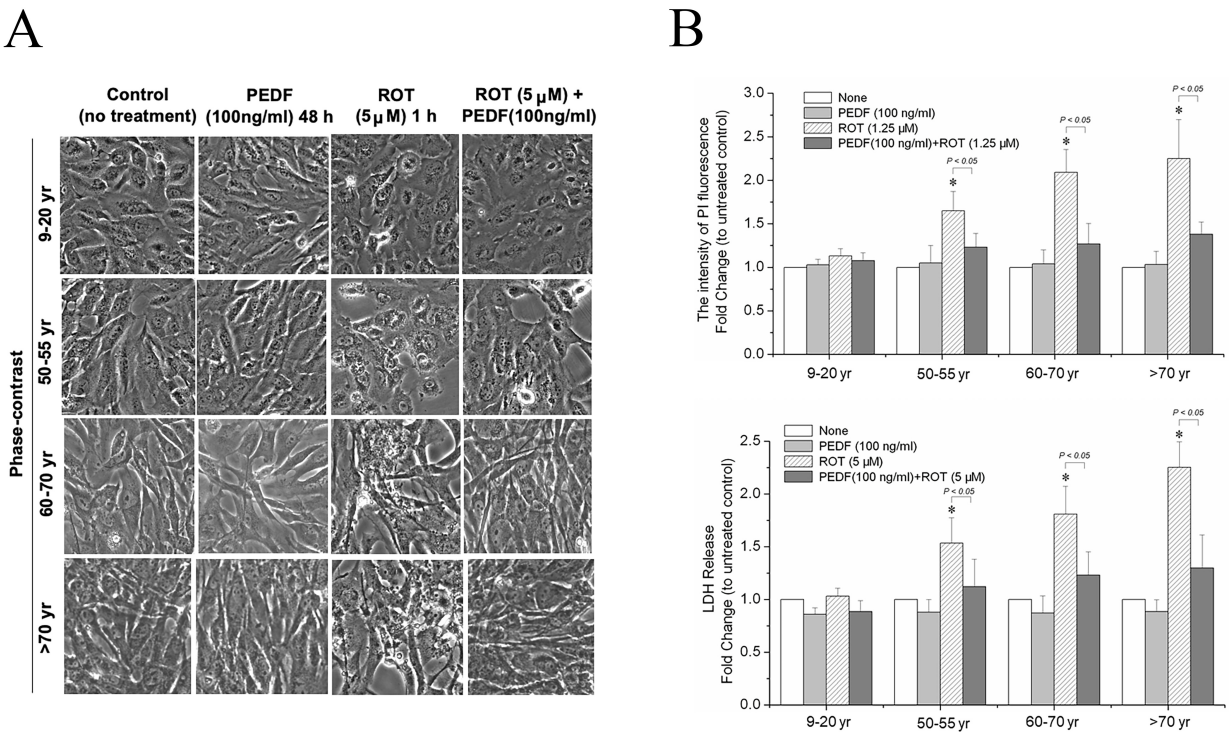

Fig. 1. Pigment epithelium-derived factor (PEDF) protects aging retinal pigment epithelium (RPE) cells from rotenone (ROT) toxicity. (A) Phase-contrast micrographs of primary cultures of human RPE cells, showing that RPE cells obtained from 9–20 and 50–55-year-old donors were round and more regularly shaped compared to the elongated features of those obtained from the 60–70 and >70 years age groups. After treatment with 5 μM ROT for 1 h, the aged RPE cells had a generally unhealthy and degenerative appearance. Fewer cells were observed in the aging group after ROT treatment, partly due to cell detachment and, in part, cell death. RPE cells from 9–20-year-old donors maintained a relatively healthy appearance, even in the presence of ROT. Pretreatment with PEDF (100 ng/mL) for 48 h blocked the effects of ROT on aged RPE cells (60–70 and >70 years). Scale bar = 30 μm. (B) PEDF prevents ROT-induced RPE cell death. The histogram illustrates the relative percentage of cell death in cultures after ROT treatment and protection by PEDF. (Upper) Relative amount of PI fluorescence intensity in RPE cultures determined by flow cytometry. Treatment with 1.25 μM ROT for 24 h caused an increase in PI levels in aged RPE cultures (50–55, 60–70, and >70 years) but had minimal effect on the cultures of the 9–20 years age group (1×10^6 cells were used). Pretreatment of RPE cells with 100 ng/mL PEDF for 48 h prevented the increase in PI levels when the cells were treated with ROT, as was evident in cultures from donors aged 50–55, 60–70, and >70 years. (Lower) Increase in LDH release after 1 h of ROT treatment. The fold-changes in LDH release were 1.54 (± 0.24), 1.81 (± 0.26), and 2.23 (± 0.24) for the 50–55, 60–70, and >70 years age groups, respectively, indicating that aged RPE cells were more susceptible to death caused by ROT-induced toxicity. Pretreatment of cells with PEDF (100 ng/mL) for 48 h significantly reduced LDH release into the cytoplasm after ROT treatment compared with untreated cells. The fold-changes in LDH release were 1.12 (± 0.26), 1.23 (± 0.22), and 1.30 (± 0.31), for the 50–55, 60–70, and >70 years age groups, respectively. Data are presented as fold-changes in PI intensity or LDH release from treated RPE cells compared to untreated control cells. The results are expressed as the mean ± S.E. of three independent experiments, each performed in triplicate. * Indicates a significant difference from the untreated control RPE cells set at $p < 0.05$.

min. The reaction was terminated by adding a stop solution, and the absorbance was measured at 490 nm using the Benchmark Microplate Reader. The extent of cell death was estimated as a percentage based on LDH activity, with an untreated internal control serving as a reference for total cell death.

2.6 Electron Microscopy

The cells were seeded in 6-well plates with specific substrates in the culture media and incubated for 48 h. For primary fixation, the RPE cells were treated with a solution of 2% glutaraldehyde and 85 mM cacodylate buffer for 3 h at room temperature. For secondary fixation, the cells were treated with 1% buffered osmium tetroxide at 4 °C overnight. Following dehydration in a graded series of ethanol solution, the samples were infiltrated with propylene oxide and EMbed 812 (1:1) and allowed to rotate overnight before embedding in 100% EMbed 812. Semithin sections were obtained, stained with toluidine blue and examined under a light microscope. If the sections appeared satisfactory under light microscopy, the EMbed 812 blocks were mounted and sectioned on a Sorvall MT2-B ultrami-

crotome at a thickness of 0.08 µm (800 Å) in preparation for electron microscopy. The ultrathin sections were mounted on square 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined using a Philips 400 transmission electron microscope [46,47].

2.7 Morphological Analysis of Mitochondria in RPE Cultures

The morphology of the mitochondria and mitochondrial cristae was observed at a magnification of 35,000×. Morphometric analysis was performed by two experienced observers at our core facility using the NIH ImageJ program (version 1.48; National Institute of Health, Bethesda, MD, USA). In cases where discrepancies arose, a third observer re-evaluated the samples.

2.8 Measurement of Reactive Oxygen Species

Cellular oxidative stress is determined by the amount of ROS in the cytoplasm [48,49]. Following trypsinization, RPE cells were harvested and incubated at a concentration of 2×10^6 cells/mL with freshly prepared ROS indicator H2-DCF-DA in serum-free media in the dark at 37 °C. H2-DCF-DA penetrates the cells and emits green fluorescence upon oxidation by reacting with H2O2 and, to a certain extent, NO. To obtain stable and reproducible results, we used 0.4 µM H2-DCF-DA for 30 min for flow cytometry measurements. The treated cells were rinsed twice with PBS to remove excess H2-DCF-DA, and the samples were immediately analyzed by flow cytometry using the FL channel (excitation wavelength: 488 nm, emission wavelength: 530 nm). Flow cytometric analyses were performed using a flow cytometer (BD FACS Aria™, Becton Dickinson, Franklin Lake, NJ, USA). At least 10,000 cells from each donor were analyzed, and the data were processed using the FCS Express software. The results were expressed as the median fluorescence intensity in arbitrary units, calculated from the average of triplicate measurements for each donor sample.

2.9 Measurement of Cellular ATP

The ATP levels were determined using a luciferin/luciferase-based ATP assay. Briefly, cells were grown in 96-well plates and untreated and pretreated (100 µg/mL cells) RPE cells were exposed to 1.25 µM ROT in 50 µL of culture medium for 24 h and 48 h, respectively. After treatment, the cell membranes were permeabilized using 50 µL of somatic cell ATP-releasing reagent (FL-SAR; Sigma-Aldrich) and allowed to react with 50 µL of ATP Assay Mix Reagent (FLAA; Sigma-Aldrich) containing luciferin and luciferase. Luminescence was immediately measured using the Orion II Luminometer (Berthold Detection Systems, Oak Ridge, TN, USA). Cellular ATP levels were expressed as the fold change in luminescence intensity compared to that of untreated RPE control cells.

2.10 Measurement of Mitochondrial Membrane Potential ($\Delta\Psi m$)

The $\Delta\Psi$m indicator, JC-1, was used to evaluate changes in $\Delta\Psi$m in RPE cells of various ages. JC-1 is a lipophilic cationic dye that permeates the plasma and mitochondrial membranes. The dye fluoresces red upon aggregation in the matrix of healthy high-potential mitochondria, whereas it fluoresces green in cells with low $\Delta\Psi$m. JC-1 was freshly diluted in serum-free DMEM to a final concentration of 1 µg/mL and added to suspensions of treated or non-treated cells at a density of 2×10^6 cells/mL. The samples were incubated for 20 min at 37 °C in the dark, rinsed twice with PBS, and immediately analyzed using flow cytometry at an excitation wavelength of 488 nm. Data were collected at emission wavelengths of 530 nm for green fluorescence and 590 nm for red fluorescence. The results were expressed in arbitrary units as median fluorescence intensity.

2.11 Measurement of Calcium Levels in the Cytoplasm ($[Ca^{2+}]_c$) and Mitochondria ($[Ca^{2+}]_m$) of RPE Cells

Changes in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were measured using the fluorescent probes fluo-3/AM and Rhod-2/AM (Kd ~570 nM), respectively, as previously described [50,51]. Fluorescence intensity was measured using flow cytometry andconfocal microscopy. For flow cytometry analysis, the cells were cultured in 6-well plates at a density of 1×10^5 cells/well. After some cells were exposed to 5 µM ROT for 1 h only, or treated RPE cells with 100 µg/mL PEDF for 48 h only, or pretreated RPE cells with 100 µg/mL PEDF for 48 h before 5 µM ROT was added for 1 h, cells were loaded with either 1 µM fluo-3/AM for 30 min, or 1 µM rhod-2/AM for 1 h. The cells were then trypsinized, washed twice with cold PBS, resuspended in 200 µL PBS, and immediately analyzed by flow cytometry (fluo-3/AM, excitation wavelength: 488 nm, emission wavelength: 525 nm; Rhod-2/AM, excitation wavelength: 549 nm, emission wavelength: 581 nm). The fluorescence intensity of 10,000 labeled cells was routinely collected for each analysis, and the data were expressed as the median fluorescence intensity in arbitrary units, calculated from the average of at least three separate experiments.

2.12 RT- and Real-Time PCR

Total mRNA from primary cultures of human RPE cells was isolated using an RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) and RT-PCR was carried out using iTaq polymerase (Bio-Rad) with an annealing temperature of 58 °C for 35 cycles to amplify retinol dehydrogenase (RDH). The primer sequences for RDH were 5′-GGCATTGTTGATTAGGATGG-3′ (sense) and 5′-GCTCTTAGCCTTGCAGTTTG-3′ (antisense). GAPDH was used as the internal RNA loading control, and sam-

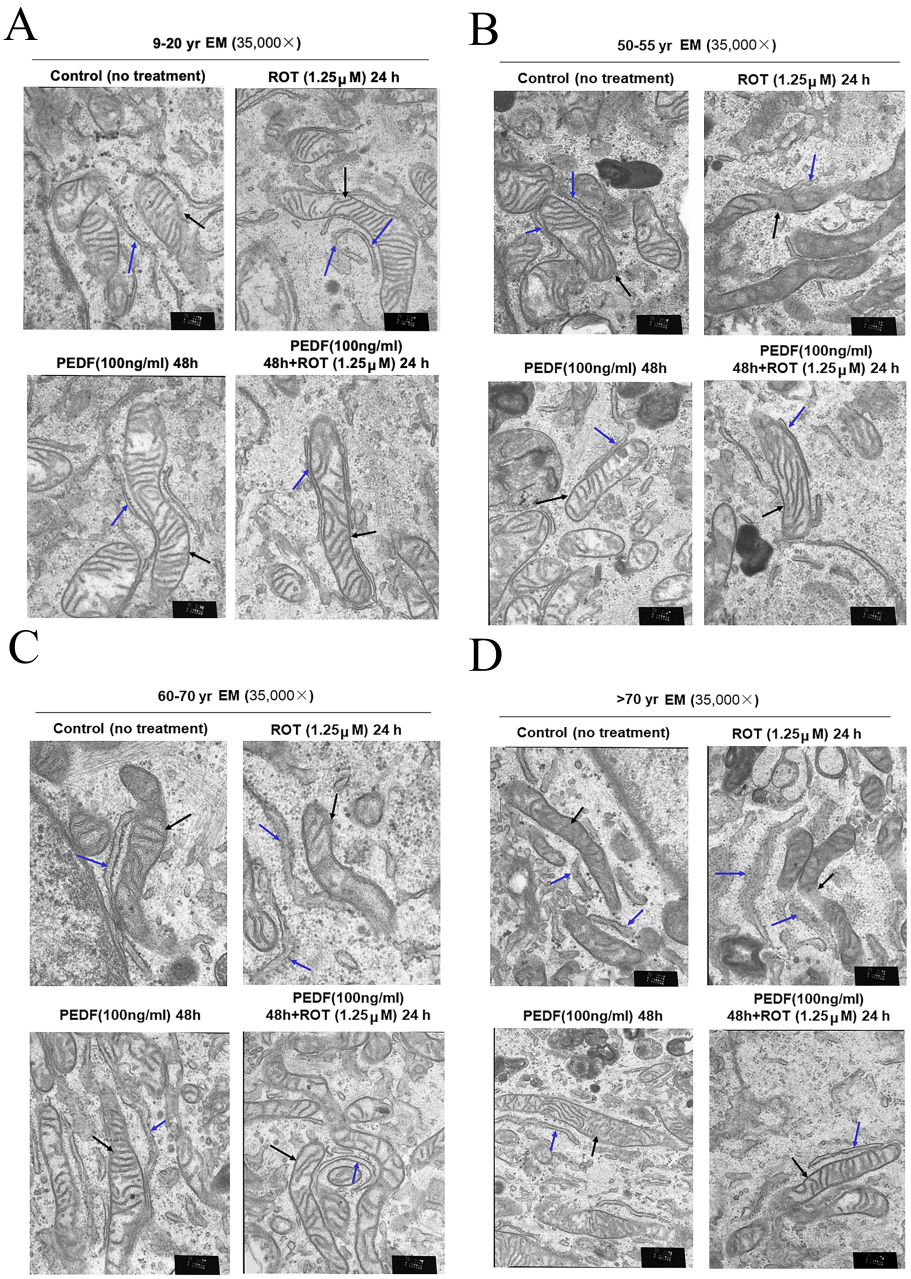

Fig. 2. Electron micrographs (magnification: 35,000×) showing that the mitochondria appear abnormal in RPE cells from aged donors and that PEDF preserves mitochondrial structural integrity, even in the presence of ROT (A–D). Mitochondria in cells from donors aged 9–20 and 50–55 years are regular and oval shaped, and have intact cristae. In contrast, the mitochondria in cells from donors aged 60–70 and >70 years are elongated and irregularly shaped. These mitochondria also demonstrate a loss of cristae integrity and highly electron-dense matrices. Treatment with ROT (1.25μM for 24 h) induced aging-like elongation in the mitochondria of cells from 9–20-year-old donors. However, the finer cristae structures remain clear, and there is minimal change in the matrix. In contrast, mitochondria in cells from older donors (50–55, 60–70, and >70 years) exhibit fragmentation, swelling, and unclear cristae structures after ROT treatment. Pretreatment with PEDF (100 ng/mL for 48 h) preserved the mitochondrial structure during ROT exposure. This is especially evident in mitochondria in cells from older donors (50–55, 60–70, and >70 years), where the cristae structure was more clearly defined and the matrix less electron-dense in the PEDF-treated samples. These results indicate that the mitochondria in aged RPE cells are more sensitive to ROT than younger cells, and that PEDF can protect mitochondria from ROT-induced damage. Scale bar = 1.5 μm; black arrows: mitochondria; blue arrows: ER.

ples where no reverse transcriptase was added were used as negative controls to ensure that amplification was RNA-dependent. The PCR products were resolved by 1% agarose gel electrophoresis. For quantitative real-time PCR, the

two-step amplification protocol was performed using the iQ SYBR green supermix solution (Bio-Rad). Both melting curve and gel electrophoretic analyses were used to determine amplicon homogeneity and data quality.

2.13 Statistical Analysis

Data were statistically analyzed via SPSS 23.0 Software (SPSS, Inc., Chicago, IL, USA). All assays were performed using at least 3 repeated experiments in triplicates and data were expressed as means $\pm$ standard error (S.E.). Multi-group comparisons were done utilizing a one-way ANOVA and using an SNA-Q test for pairwise comparison between two groups. Statistical significance was set at $p < 0.05$.

3. Results

Cumulative oxidative damage to the RPE causes tissue degeneration and is the primary pathology of AMD [9–11]. Based on previous experimental evidence supporting a link between mitochondrial dysfunction, ER stress, and age-related degenerative diseases, we studied mitochondrial structure and function in human RPE cells obtained from donors of different age groups, including young, adult, and older individuals. In our previous study, we provided ultrastructural and biochemical evidence for mitochondrial decay in aging human RPE cells. The mitochondria of these cells exhibited fragility and swelling and were elongated and tubular in structure. Moreover, we observed alterations in matrix density and poorly defined intramembranous cristae within the mitochondria. Additionally, there was a significant loss of mitochondria and decreased ATP and ROS levels. The concentration of $[Ca^{2+}]_c$ decreased, while that of $[Ca^{2+}]_m$ increased. Moreover, the aging RPE cells exhibited decreased $\Delta\Psi m$ and increased susceptibility to H2O2 toxicity. These findings correlated with the morphological changes observed in primary RPE cell cultures. Here, we present evidence that PEDF protects mitochondrial function in aging RPE cells, including decreased ROS, $[Ca^{2+}]_c$, and $[Ca^{2+}]_m$ levels, and increased ATP generation, $\Delta\Psi m$, and UCP2 expression in RPE cells. All experiments were performed in triplicate and repeated three different times for each donor sample.

3.1 PEDF Protects Aging RPE Cells from ROT Toxicity

Fig. 1A shows light micrographs of primary cultures of human RPE cells obtained from donors of different ages. Notable morphological differences were observed between the monolayer cultures of younger (9–20 and 50–55 years) and aged (60–70 and >70 years) RPE cells. The younger RPE cells were more regular and cuboidal-like in shape and formed tight monolayers compared to the aged cells. With increasing chronological age, the cells appeared more elongated and fibroblast-like and did not form a complete monolayer, even after extended culture time. There were areas in the culture dish where aged cells did not migrate and populate, possibly because of a growth-negative secretory product from cells in those areas or a diminished migration capacity resulting from alterations in adhesion properties. After treatment with 5 µM ROT for 1 h, aged RPE cells showed a general unhealthy and degenerative appearance. Fewer cells were observed in the aging group after ROT treatment, owing to cell detachment and, in part, cell death. In contrast, RPE cells from 9–20-year-old donors maintained a relatively healthy appearance, even in the presence of ROT. Pretreatment with PEDF (100 ng/mL) for 48 h mitigated the effects of ROT on aged RPE cells. These results indicated that aged RPE cells were more susceptible to ROT-induced toxicity than younger cells and that PEDF provided protection to aged RPE cells.

Fig. 1B shows that PEDF prevented ROT-induced RPE cell death. The histogram shows the relative percentages of cell death in cultures after ROT treatment, with and without PEDF protection. Treatment with 1.25 µM ROT for 24 h led to increased PI levels in aged RPE cultures (50–55, 60–70, and >70 years) but had minimal effect on the younger cultures (9–20 years) (1×10^6 cells were used). Retreatment of RPE cells with 100 ng/mL PEDF for 48 h prevented the elevation of PI levels when the cells were treated with ROT. This protective effect was observed in RPE cultures in aged individuals. Upon ROT treatment for 1 h, there was a significant increase in LDH release from the RPE cells with fold-changes of 1.54 ($\pm$ 0.24), 1.81 ($\pm$ 0.26), and 2.23 ($\pm$ 0.24) for the 50–55, 60–70, and >70 years age groups, respectively. This indicated that the aged RPE cells were more susceptible to death by ROT than the younger cells. In contrast, pretreating the cells with PEDF (100 ng/mL) for 48 h significantly reduced LDH release into the cytoplasm after ROT treatment, compared to untreated cells. The fold-changes in LDH release were 1.12 ($\pm$ 0.26), 1.23 ($\pm$ 0.22), and 1.30 ($\pm$ 0.31) for the 50–55, 60–70, and >70 years age groups, respectively. Notably, the effects of PEDF-pretreatment on cells from individuals aged 9–20 years were minimal after ROT treatment.

3.2 Ultrastructural Differences are Evident in the Mitochondria of RPE Cells with Aging after ROT and PEDF Treatment

Comparison of electron microscopic images of cultures obtained from donors of different chronological ages revealed significant variations in mitochondrial morphology after ROT and PEDF treatment. Fig. 2 demonstrates that PEDF preserved the mitochondrial structural integrity, even in the presence of ROT. Electron micrographs (magnification: 35,000×) of primary RPE cultures illustrate that mitochondria in RPE cells from the younger donors (9–20 and 50–55years) were regular and oval shaped and had intact cristae. In contrast, the mitochondria in cells from the older donors (60–70 and >70 years) were elongated, irregularly shaped, and swollen. Additionally, there was a loss of cristae integrity, and the matrices were highly

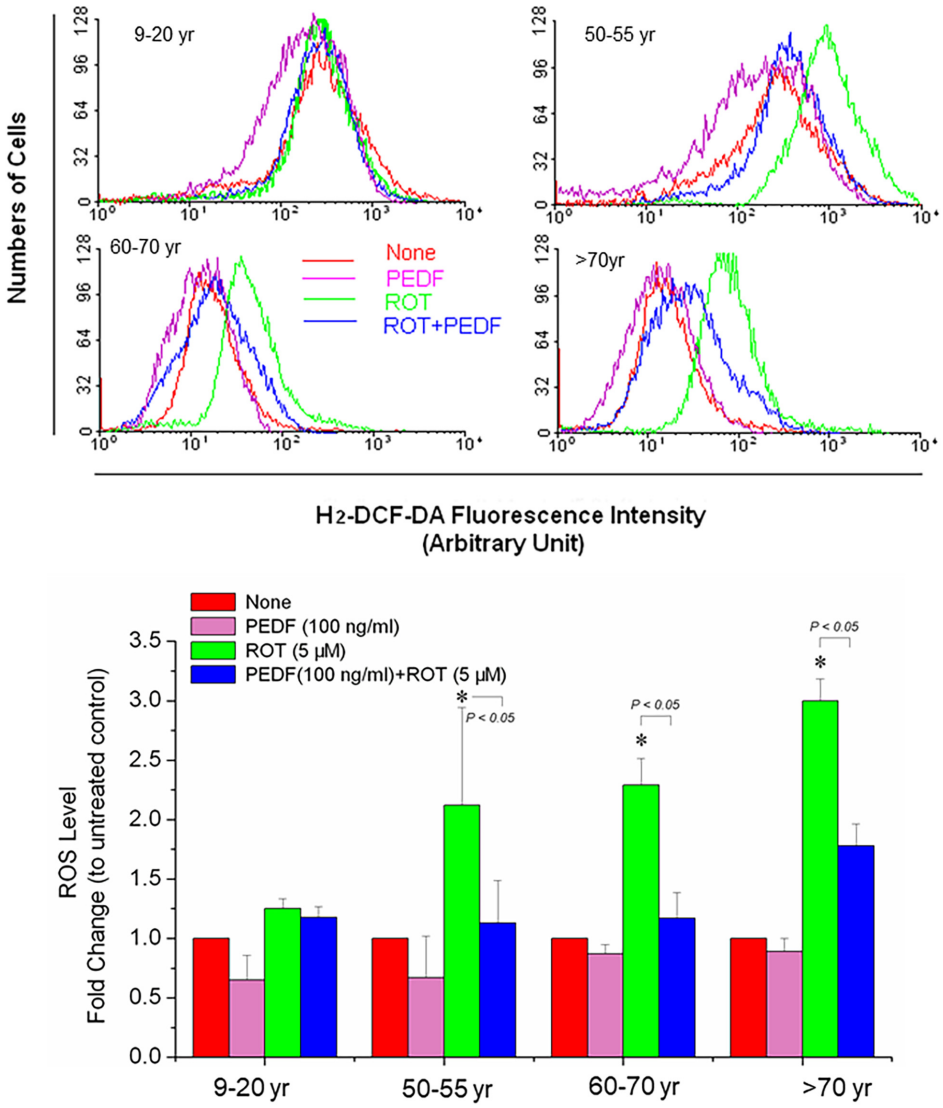

Fig. 3. PEDF reduces ROS levels in aged RPE cells. Distribution of the ROS indicator, H2-DCF-DA, fluorescence intensity in RPE cell cultures using flow cytometry. Younger RPE cultures (9–20 and 50–55 years) had stronger H2-DCF-DA fluorescence intensity than aged RPE cultures (60–70 and >70 years) (upper). The relative amount of total H2-DCF-DA fluorescence intensity in RPE cultures (lower) shows that treatment with 5 μM ROT for 1 h caused an increase in ROS level in the aged cells compared to untreated cells by 2.12-fold (± 0.82), 2.29-fold (± 0.22) and 3.0-fold (± 0.18) in cells from donors aged 50–55, 60–70 and >70 year, respectively. However, it had a minimal effect on cells from donors aged 9–20 years. Pretreatment of the cells with 100 ng/mL PEDF for 48 h prevented an increase in ROS levels induced by ROT, particularly in the cells from older donors. A fold change decrease in ROS level of 0.99, 1.12, and 1.22 was observed compared to non-pretreated cells. Data are presented as fold-changes in fluorescence intensity levels of treated RPE cells compared to untreated control cells. The results are expressed as the mean ± S.E. of repeated experiments, each performed in triplicate. * Indicates a significant difference from the untreated control RPE cells at $p < 0.05$.

electron-dense. ROT treatment (1.25 μM for 24 h) induced mitochondrial aging-like elongation in the cells of 9–20-year-old donors. However, finer cristae structures remained discernible, and minimal changes were observed in the matrix. In contrast, mitochondria in cells from the older donors (50–55, 60–70, and >70 years) demonstrated fragmentation, swelling, and unclear cristae structures following ROT treatment. Notably, pretreatment with PEDF (100 ng/mL for 24 h) preserved the mitochondrial structure in the presence of ROT. This was particularly evident in the RPE cells from the older donors (50–55, 60–70, and >70 years), where cristae structures were more clearly defined and the matrix exhibited lower electron densities in the PEDF-treated samples. These results indicate that mitochondria in aged RPE cells are more susceptible to ROT-induced damage than younger cells and that PEDF provided protection against ROT-induced damage to the mitochondria.

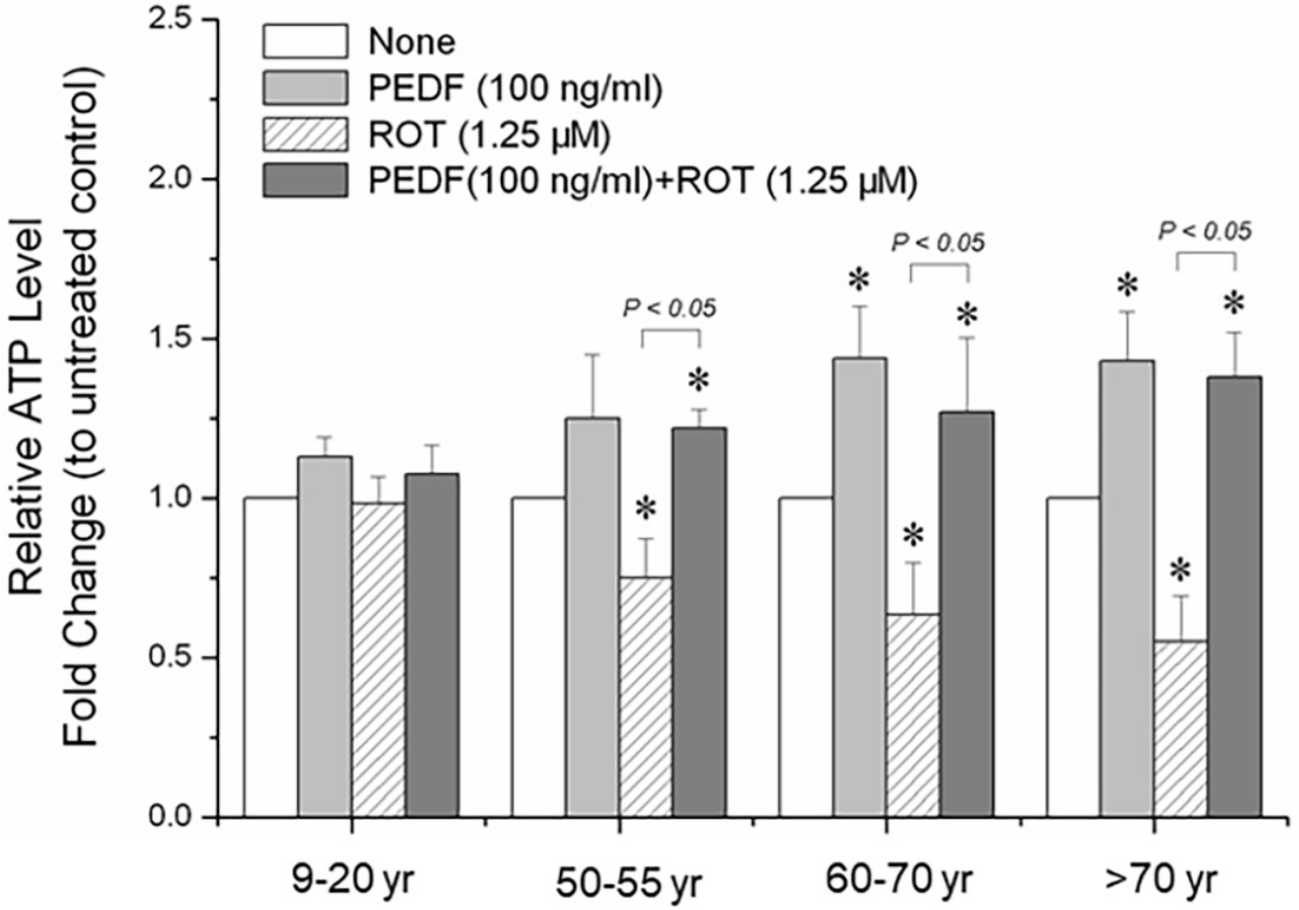

Fig. 4. ATP levels decrease with increasing RPE cell aging. The luciferin/luciferase-based ATP Assay results demonstrated that the ATP levels in RPE cells from donors aged 50–55, 60–70, and >70 years were 31%, 35%, and 45% lower than those in the cells from 9–20-year-old donors, respectively. Treatment with 1.25 µM ROT for 24 h resulted in a further decrease in ATP levels by 0.75-fold (± 0.12), 0.64-fold (± 0.16), and 0.55-fold (± 0.14) in cells from donors aged 50–55, 60–70, and >70 years, respectively. However, ROT treatment had minimal effects on RPE cells from the younger donors (9–20 years). Pretreatment with PEDF (100 ng/mL for 48 h) increased the ATP levels by blocking the ROT effects on ATP levels. This was particularly evident in the cells from older donors aged 50–55, 60–70, and >70 years which showed a 1.63-, 1.98-, and 2.51-fold increase in ATP levels, respectively, compared to non-pretreated cells. Data are presented as fold-changes in fluorescence levels of treated RPE cells compared to untreated control RPE cells. The results are expressed as the mean ± S.E. of three repeated experiments, each performed in triplicate. * Indicates a significant difference from the untreated control RPE cells set at $p < 0.05$.

3.3 PEDF Reduces ROS Levels in Aged RPE Cells

The RPE cultures from younger donors (9–20 and 50–55 years) exhibited stronger H2-DCF-DA fluorescence intensity than those from older donors (60–70 and >70 years). The relative total H2-DCF-DA fluorescence intensity in RPE cultures is presented in Fig. 3 (lower panel). Treatment with 5 µM ROT for 1 h resulted in an increase in ROS levels in aged cells compared to untreated cells. The fold-changes in ROS level were 2.12 (± 0.82), 2.29 (± 0.22), and 3.0 (± 0.18) in cells from donors aged 50–55, 60–70, and >70 years, respectively. However, this effect was minimal in cells from 9–20-year-old donors. Pretreatment of the cells with 100 ng/mL PEDF for 48 h prevented a rise in ROS levels induced by ROT, particularly in the cells from the donors aged 50–55, 60–70, and >70 years. The PEDF-pretreatment resulted in a decrease of 0.99-, 1.12-, and 1.22-fold compared to non-pretreated cells (Fig. 3). The experiments were repeated thrice in triplicate ($p < 0.05$).

3.4 PEDF Increased Endogenous ATP Levels in Aging RPE Cells

The results presented in Fig. 4 demonstrate a decrease in ATP levels with increased aging of the RPE cells. ATP levels in cells from donors aged 50–55, 60–70, and >70 years were 31%, 35%, and 45% lower, respectively, than in cells from 9–20-year-old donors ($p < 0.05$). Treatment with 1.25 µM ROT for 24 h resulted in a further decrease in ATP levels by 0.75-fold (± 0.12), 0.64-fold (± 0.16), and 0.55-fold (± 0.14) in cells from donors aged 50–55,

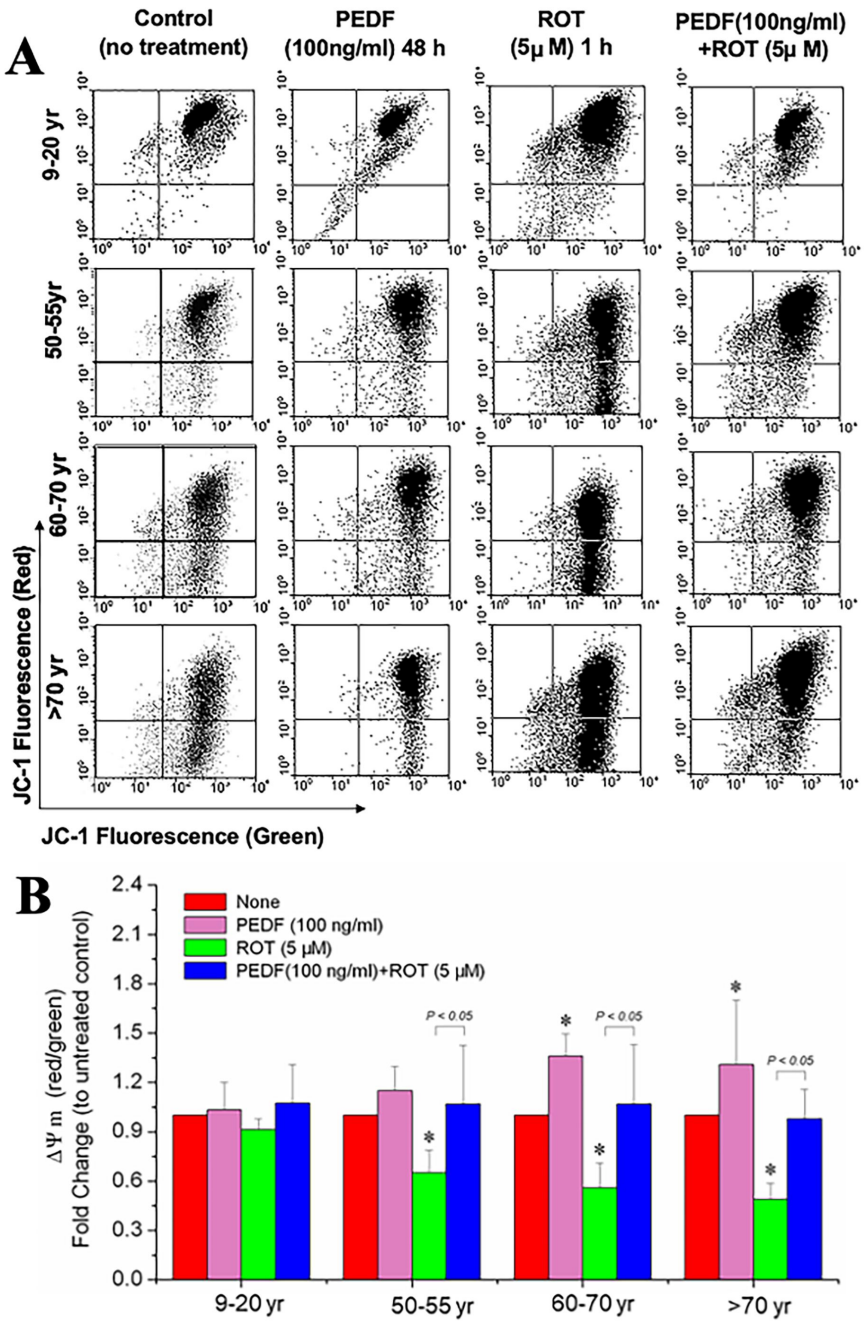

Fig. 5. Mitochondrial membrane potential (ΔΨm) decreases with increasing RPE cell aging. ΔΨm was examined using flow cytometry and the fluorescence indicator JC-1. The ΔΨm is 1.2-fold (± 0.1), 1.52-fold (± 0.2), and 2.1-fold (± 0.3) lower in RPE cells from donors aged 50–55, 60–70, and >70 years after 5 μM ROT treatment, respectively, compared to RPE cells from donors aged 9–20. Relative amount of red/green fluorescence intensity ratio in RPE cultures. 5 μM ROT treatment for 1 h leads to an additional decline in ΔΨm by 0.65 fold (± 0.13), 0.56 fold (± 0.15), and 0.49 fold (± 0.10) in RPE cells from donors aged 50–55, 60–70 and >70 years, respectively, compared to non-treated cells. Pretreatment of the cultures with 100 ng/mL PEDF for 48 h prevented the decrease in ΔΨm induced by ROT, especially in RPE cells from donors aged 50–55, 60–70 and >70 years, respectively, by 1.65-, 1.91- and 2.00-fold compared to ROT treatment alone. Results are expressed as the mean fold decrease in fluorescence levels in untreated samples ± S.E. of all experiments performed in triplicates. * Indicates a significant difference from the untreated control RPE cells at $p < 0.05$.

60–70, and >70 years, respectively. However, treatment with ROT had a minimal effect on RPE cells from 9–20-year-old donors. Pretreatment with PEDF (100 ng/mL for 48 h) increased the ATP levels. Thus, PEDF effectively counteracted the effects of ROT on ATP levels, particularly in cells from donors aged 50–55, 60–70, and >70 years. In

these groups, 1.63-, 1.98-, and 2.51-fold increases in ATP levels were observed compared to non-pretreated cells.

3.5 PEDF Increased Mitochondria Membrane Potential ($\Delta\Psi m$) in Aging RPE Cells

Given the lower bioenergetic profiles observed with increased aging, we measured the $\Delta\Psi$m in cells from different age groups. The results revealed a decrease of 1.2-fold ($\pm$ 0.1), 1.52-fold ($\pm$ 0.2), and 2.1-fold ($\pm$ 0.3) in RPE cells from donors aged 50–55, 60–70, and >70 years after 5 µM ROT treatment, respectively, compared to RPE cells from donors aged 9–20 years (Fig. 5). These findings provide compelling evidence of increased mitochondrial depolarization and impaired mitochondrial function across different chronological ages in RPE cells. Furthermore, treatment with 5 µM ROT for 1 h resulted in an additional decline in the $\Delta\Psi$m. Specifically, there was a decrease of 0.65-fold ($\pm$ 0.13), 0.56-fold ($\pm$ 0.15), and 0.49-fold ($\pm$ 0.10) in $\Delta\Psi$m in RPE cells from donors aged 50–55, 60–70, and >70 years, respectively, compared to untreated cells. Pretreatment of cultures with 100 ng/mL PEDF for 48 h prevented the decrease in $\Delta\Psi$m induced by ROT, particularly in RPE cells from donors aged 50–55, 60–70, and >70 years. In these age groups, PEDF exhibited a protective effect, resulting in $\Delta\Psi$m values that increased by 1.65, 1.91, and 2.00-fold, in cells from donors aged 50–55, 60–70, and >70 years, respectively, compared to non-pretreated cells.

3.6 Aged RPE Cells (60–70 and >70 yrs) have Lower Levels of Calcium in the Cytoplasm ($[Ca^{2+}]_c$) and Higher Mitochondria ($[Ca^{2+}]_m$) Compared to 9–20 yr

To assess calcium concentrations, we measured $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ in RPE cells using the fluorescent Ca^{2+} indicators fluo-3/AM (Fig. 6A) and Rhod-2/AM (Fig. 6B), respectively, with flow cytometry. A significant decrease in $[Ca^{2+}]_c$ and an increase in $[Ca^{2+}]_m$ was observed in the aged RPE cells compared to the cells from 9–20-year old donors. Pretreatment with PEDF decreased the $[Ca^{2+}]_c$ concentration in aged RPE cells. The relative amount of total fluo-3/AM fluorescence intensity in RPE cultures, following treatment with 5 µM ROT for 1 h, resulted in a decrease in $[Ca^{2+}]_c$ by 0.77-fold ($\pm$ 0.22), 0.56-fold ($\pm$ 0.14), and 0.57-fold ($\pm$ 0.17) in RPE cells from donors aged 50–55, 60–70, and >70 years, respectively, compared to non-treated cells. However, pretreatment of the cultures with 100 ng/mL PEDF for 48 h further decreased the $[Ca^{2+}]_c$ induced by ROT, particularly in RPE cells from donors aged 50–55, 60–70, and >70 years, by 0.51-fold ($\pm$ 0.29), 0.34-fold ($\pm$ 0.08), and 0.36-fold ($\pm$ 0.08), respectively, compared to non-pretreated cells (Fig. 6A, lower). Furthermore, the distribution of $[Ca^{2+}]_m$, as indicated by the Rhod-2 fluorescence intensity in RPE cell cultures using flow cytometry, revealed that RPE cultures from younger donors (9–20 and 50–55 years) exhibited lower Rhod-2 fluorescence signals than those from older donors (60–70 and >70 years). Pretreatment with PEDF decreased the $[Ca^{2+}]_m$ in aged RPE cells (Fig. 6B, upper). The relative amount of total Rhod-2 fluorescence intensity in RPE cultures, following treatment with 5 µM ROT for 1 h, resulted in an increase in $[Ca^{2+}]_m$ by 1.55-fold ($\pm$ 0.91), 1.75-fold ($\pm$ 0.85), and 1.81-fold ($\pm$ 0.94) in RPE cells from donors aged 50–55, 60–70, and >70 years, respectively, compared to non-treated cells. However, pretreatment of the cultures with 100 ng/mL PEDF for 48 h decreased ROT-induced $[Ca^{2+}]_m$, especially in RPE cells from donors aged 50–55, 60–70, and >70 years, by 2.18-, 2.22-, and 2.23-fold, respectively, compared to non-pretreated cells (Fig. 6B, lower).

3.7 PEDF Increases RDH Expression in aged RPE Cells

As shown in Fig. 7A,B, there was a decreased expression of RDH in aged RPE cells, as determined by RT-and real-time PCR. Pretreatment with PEDF (100 ng/mL for 48 h) significantly increased the mRNA level of RDH in aged RPE cells.

3.8 PEDF Regulates ER Stress-Related Genes Induced by ROT

As shown in Fig. 7C,D, after RPE cells were treated with ROT (1.25 µM for 24 h), the expression of ER stress-related genes, including *IP3R*, *RyR3*, *HERK*, *APP*, *GRP*, *PARK*, *GADD34*, *PERK*, *CHOP*, and *IRE1* were increased. However, the expression of these ROT-induced ER stress-related genes decreased significantly in cells pretreated with PEDF (100 ng/mL for 48 h), indicating a protective effect.

4. Discussion

The pigment epithelium is located in the retina and is constantly exposed to ROS and waste products from photoreceptors. Accumulative oxidative damage to the RPE can lead to tissue degeneration [9–11] and may be the primary underlying cause of certain visual disorders. Given the presence of experimental evidence supporting a link between mitochondrial dysfunction, aging, and several age-related degenerative diseases, we examined the structure and function of the mitochondria in primary cultures of human RPE cells obtained from donors of varying ages. In a previous study, we demonstrated that, as human RPE cells undergo normal aging, the number of mitochondria decreases and mitochondrial dysfunction increases, including numeric loss of mitochondria and lower levels of ATP and ROS. Additionally, we observed a lower $[Ca^{2+}]_c$ and higher $[Ca^{2+}]_m$, decreased $\Delta\Psi$m, and greater susceptibility to H2O2 toxicity. Here, we provided evidence that PEDF protects mitochondrial function in aging RPE cells. This evidence included decreased ROS levels and $[Ca^{2+}]_c$, and increased $[Ca^{2+}]_m$, ATP generation, and $\Delta\Psi$m. Additionally, enhanced expression of UCP2 and RD was observed in aged RPE cells. Our study provides evidence for bioenergetic deficiencies in aging RPE cells, a condition that may

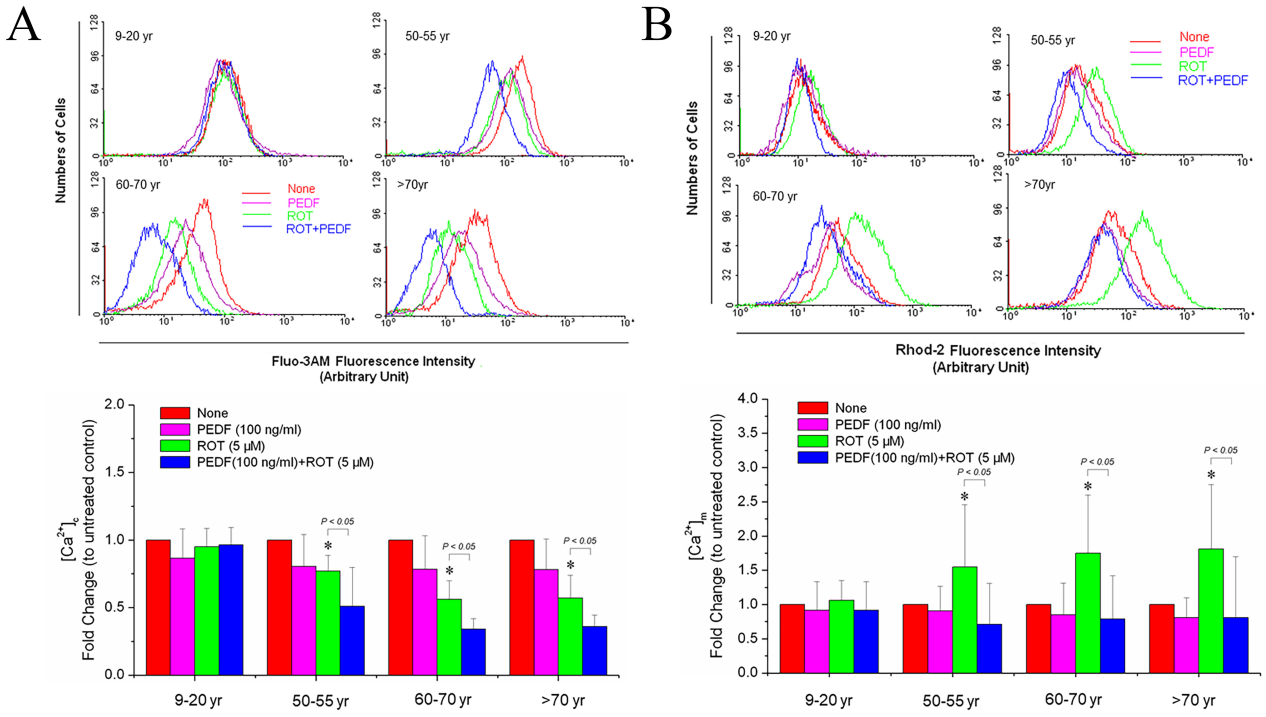

Fig. 6. PEDF decreases the level of calcium in the cytoplasm ($[Ca^{2+}]_c$) and mitochondria ($[Ca^{2+}]_m$) in aged RPE cells (60–70 and >70 years) compared to cells from donors aged 9–20 years. (A upper) Distribution of the $[Ca^{2+}]_c$ indicated, fluo-3AM fluorescence intensity in RPE cell cultures using flow cytometry. Younger RPE cultures (9–20 and 50–55 years) have stronger fluo-3AM fluorescence signal than aged RPE cultures (60–70 and >70 years). Pretreatment with PEDF decreases $[Ca^{2+}]_c$ levels in aged RPE cells. (A lower) Relative amount of total fluo-3AM fluorescence intensity in RPE cultures showing 5 μM ROT treatment for 1 h leads to a decline in $[Ca^{2+}]_c$ by 0.77-fold (± 0.22), 0.56-fold (± 0.14) and 0.57-fold (± 0.17) in 50-55, 60-70 and >70 yr RPE cells compared to non-treated cells. Pretreatment of the cultures with 100 ng/mL PEDF for 48 h also further the decreased in $[Ca^{2+}]_c$ induced by ROT, especially in RPE cells from donors aged 50–55, 60–70, and >70 years by 0.51-fold (± 0.29), 0.34-fold (± 0.08), and 0.36-fold (± 0.08) compared to non-treated cells. (B upper) Distribution of the $[Ca^{2+}]_m$ indicated the Rhod-2 fluorescence intensity in RPE cell cultures using flow cytometry. Younger RPE cultures (9–20 and 50–55 years) have lower Rhod-2 fluorescence signal than aged RPE cultures (60-70 and >70 yr). Pretreatment with PEDF decreases the level of $[Ca^{2+}]_m$ in aged RPE cells. (B lower) Relative amount of total Rhod-2 fluorescence intensity in RPE cultures showing 5 μM ROT treatment for 1 h leads to an increase in $[Ca^{2+}]_m$ by 1.55-fold (± 0.91), 1.75-fold (± 0.85), and 1.81-fold (± 0.94) in RPE cells from donors aged 52, 62, and 76 years, respectively, compared to non-treated cells. Pretreatment of the cultures with 100 ng/mL PEDF for 48 h decreased $[Ca^{2+}]_m$ induced by ROT, especially in RPE cells from donors aged 50–55, 60–70, and >70 years by 2.18-, 2.22-, and 2.23-fold decrease, respectively, compared to ROT treatment alone. Data are expressed as a fold change in fluorescence levels to 9–20 yr. Results are expressed as the mean ± S.E. of the three experiments performed in triplicate. * Indicates a significant difference from 9–20 year at $p < 0.05$.

contribute to the onset of certain retinal diseases, such as AMD. ROT, a complex I inhibitor, can exacerbate these conditions, and was used in our study to help us understand the pathology of aging in RPE cells. In contrast, PEDF can reduce the progression of this condition.

Mitochondrial structural and functional changes are commonly observed with aging. In older organisms, there is a decrease in the number of mitochondria but an increase in the organelle's size. For instance, synaptic terminals in old animals exhibit a higher percentage of oversized organelles known as megamitochondria [52]. These megamitochondria are typically found in adverse conditions and pose a serious threat to cell survival [53,54]. It is believed that the decrease in mitochondrial number loss is due to impaired duplication capacity, while the shift in size serves as a compensatory mechanism to maintain constant volume density throughout an individual's lifespan, thus increasing the mitochondrial area involved in cellular respiration [52,55–57].

We observed that, with increased chronological age, there is a marked decrease in the number of mitochondria and an increase in their size in RPE cells, which is consistent with reports on other aging tissues. Moreover, we found that treatment with ROT further exacerbated the morphological changes in mitochondria in aged RPE cells. These abnormal mitochondria had partial-to-complete loss

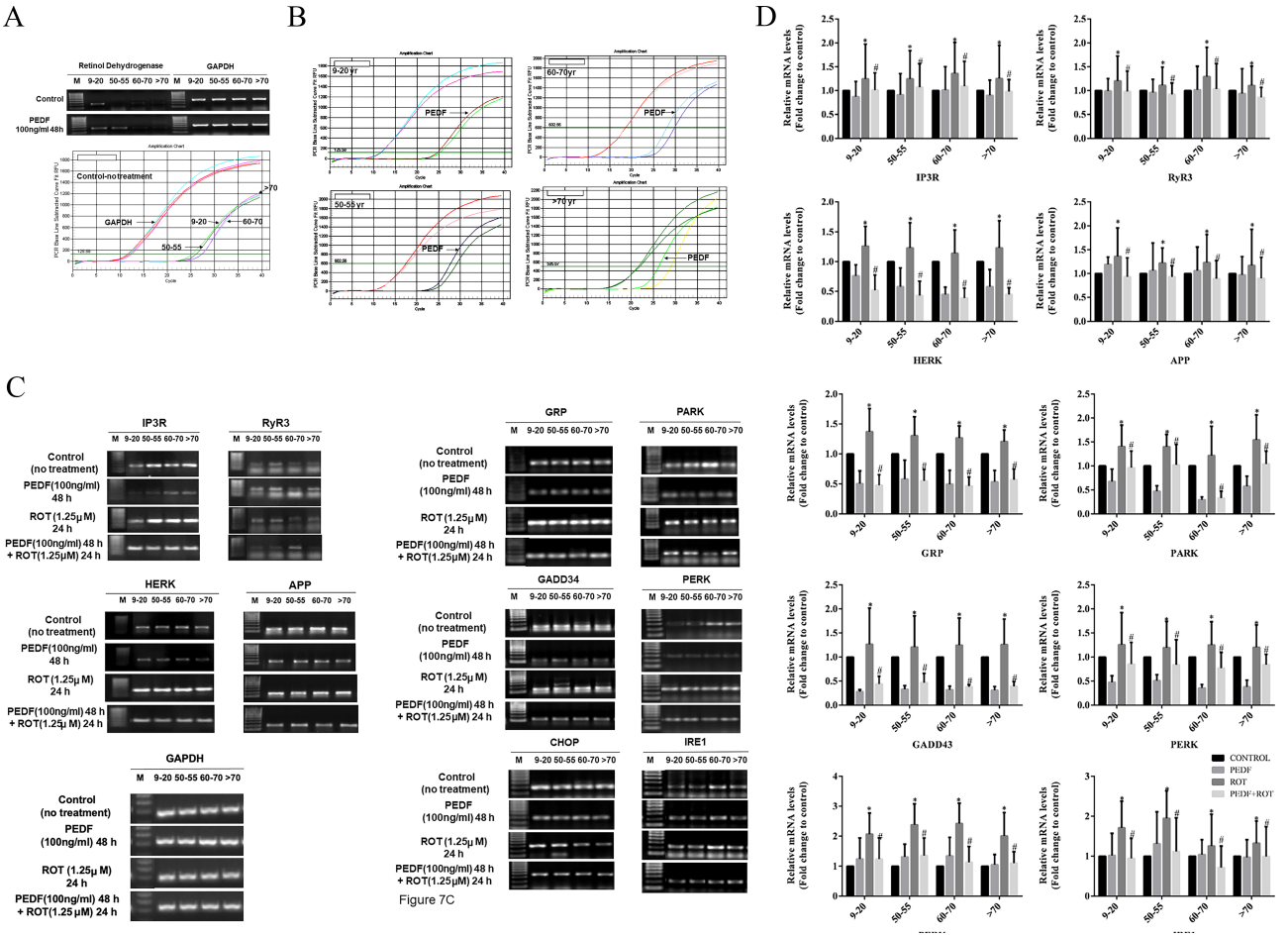

Fig. 7. PEDF increases retinol dehydrogenase (RDH) mRNA levels in aged RPE cells. (A) RT- and real-time PCR show that the expression of RDH is lower in aged RPE cells than in the cells from donors aged 9–20 years. (B) Pretreatment with PEDF (100 ng/mL for 48 h) significantly increased the mRNA level of RDH in aged RPE cells. (C,D) The expression of genes of *IP3R*, *RyR3*, *HERK*, *APP*, *GRP*, *PARK*, *GADD3*4, *PERK*, *CHOP*, and *IRE1* associated with ER stress in aged RPE cells was measured by RT-PCR. GAPDH was used as the control. *Indicates a significant difference from the control group at $p < 0.05$. # Indicates a significant difference from the ROT treated group at $p < 0.05$.

of cristae and increased matrix density. We ruled out the possibility that the morphological changes were due to fixation and processing artifacts by processing all the samples similarly. These findings are similar to those reported by Feher *et al*. [19], who showed mitochondrial abnormalities in RPE cells *in situ* with increased aging of the human retina and that this condition worsened in eyes with AMD. Based on these observations and our findings, we propose that impaired mitochondrial function or loss of this organelle renders aging RPE cells more susceptible to oxidative damage, which could be a precursor to the development of AMD. Pretreatment with PEDF protect mitochondria from morphological changes induced by ROT in aged RPE cells.

The free radical theory of aging [58] states that changes in biological function over time are due to cumulative cellular damage caused by ROS. This theory is supported by studies showing progressive, even exponential, accumulation of ROS-damaged proteins, lipids, and nucleic acids as cells and organisms age [59]. Oxidative injury to cells is associated with several diseases, including Alzheimer's disease [60], amyotrophic lateral sclerosis [61], muscular dystrophy [62], Parkinson's disease [63], AMD [64], and cataract [65]. These are all late- or slow-onset diseases in which damage accumulates over time. Mitochondrial damage has been demonstrated in many of these diseases [66], implicating this organelle as a key player in disease progression. However, we observed decreased ROS levels in the RPE cells from older donors. This contradicts popular findings indicating that RO production is increased in aged tissues. One explanation for this is that the mitochondria in these cells are less metabolically active and, therefore, produce less ROS. Our results showed that there was significantly lower ATP generation as the RPE aged, supporting this hypothesis. Less ROS production may be a mechanism for "self" preservation, potentially extending the lifespan of these cells. However, despite the re-

duced ROS production, the overall mitochondrial function impairment we observed renders these cells more susceptible to environmental challenges. Defects in complex I of the mammalian mitochondrial respiratory chain are known to be related to an increase ROS production, which is linked to several degenerative disorders [19,57]. To study whether the increase in ROS production in the aging RPE cells is a result of mitochondrial complex I defects, we used an inhibitor of mitochondrial function, including ROT (a complex I inhibitor). Our data showed that ROT triggers a rapid increase in ROS production, which exceeded the levels already present in aged RPE cells, with no significant changes in younger RPE cells (9 years), indicating a direct link between mitochondrial complex I defects and elevated ROS levels in aged RPE cells. PEDF blocks ROT-induced increase in ROS levels in aged RPE cells.

Because of its role as the major energy source of the cell, mitochondrial dysfunction underlies key events leading to apoptosis [12–14]. Some mitochondrial-specific actions leading to apoptosis include loss of $\Delta\Psi m$, induction of MPT opening, and cytosolic translocation of apoptogenic factors, such as cytochrome c [67,68]. Here, we found that RPE cells of aged donors have a lower ATP level and $\Delta\Psi m$ than those of younger donors. We showed that mitochondria were depolarized in aged RPE cells; however, there was no significant release of cytochrome c by these cells. Treatment with ROT caused further ATP depletion and mitochondrial depolarization in aged RPE cells, whereas younger RPE cells (9 years old) were relatively unaffected by ROT treatment. The increased sensitivity of aged RPE cells to mitochondrial complex I suggested the presence of an intrinsic mitochondrial complex I defect in these cells. PEDF increases ATP generation and $\Delta\Psi m$ in aged RPE cells.

Ca^{2+} plays a central role in cell signaling [69–71]. Its concentration in the cellular environment changes in response to a range of signals that allow it to modulate cellular functions. The mitochondria are complex cellular structures that participate in various intracellular processes, including cellular Ca^{2+} signaling. They can modulate the amplitude and spatiotemporal organization of cytoplasmic Ca^{2+} signals because of their ability to rapidly accumulate and release Ca^{2+} into the cytosol [50,69–72]. Mitochondria Ca^{2+} overload leads to ROS overproduction, which, in turn, triggers the MPTP opening and apoptosis mechanisms [73,74]. In a previous study, we found lower $[Ca^{2+}]_c$ and higher $[Ca^{2+}]_m$ in aged RPE cells, suggesting a possible role of Ca^{2+} dysregulation in RPE degeneration. Cells. In this study, we demonstrated Ca^{2+} dysregulation in aged RPE cells, with further decreases in $[Ca^{2+}]_c$ and increased $[Ca^{2+}]_m$ levels after ROT treatment. PEDF prevents ROT-induced increases in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ levels.

The highest level of RDH expression was observed in the retina, where it was localized to the inner segments and cell bodies of Rod and cone photoreceptors [75]. *In vitro*, RDH catalyzes the oxidoreductive interconversion of all-trans- and cis-isomers retinoids. This enzyme also catalyzes the production of medium-chain aldehydes with lower affinity. This dual specificity of the substrate leads to the following: Role of RDH in the reduction of all-trans retinal to all-trans retinol in the visual cycle [75]. Although bidirectional *in vitro*, RDH acts as a retinal reductase in living cells by shifting retinoid homeostasis. Towards increased retinol levels and decreased bioactive retinoic acid levels. RDH reductase activity protects cultured cells from death caused by the addition of exogenous retinaldehyde, and this effect is correlated with lower Levels of retinoic acid in DHH-expressing cells. However, RDH contributes to all-trans retinal clearance due to its loss results in a slightly increased accumulation of retinotoxic N-retinylidene-N-retinylethanolamine (A2E), that accumulates when all-trans retinal is not normally metabolized. In the present study, the increased expression of RDH after PEDF treatment partly explains the protective function of PEDF against mitochondrial dysfunction by reducing A2E levels.

Mitochondria-ER contact sites (MERCS) are morphofunctional units located in the tightly adherent sites of the ER. Endomembrane and outer mitochondrial membranes. MERCS is believed to play a pivotal role in several functions maintain cellular homeostasis, including mitochondrial quality control, calcium homeostasis, lipid biosynthesis, Autophagy, apoptosis, unfolded protein response, and ER stress [76–78]. According to previous studies, dysfunction in the The MERCS is associated with neurodegenerative diseases [79]. ROT treatment results in changes in intracellular mitochondria-ER contact sites, leading to ER stress [79,80]. Similar to the results of our study, ROT-induced ER stress and Increased expression of ER stress-related genes such as *IP3R*, *RyR3*, *HERK*, *APP*, *GRP*, *PARK*, *GADD34*, and *PERK CHOP* and *IRE1*. However, PEDF treatment decreased the expression of ROT-mediated ER stress-related genes. It further suggested that PEDF protects ROT-damaged RPE cells by protecting mitochondrial and ER functions.

5. Conclusions

In conclusion, we present strong evidence that PEDF protects mitochondrial and ER function in aging RPE cells, including decreased ROS, $[Ca^{2+}]_c$, and $[Ca^{2+}]_m$ levels, increased ATP generation, $\Delta\Psi m$, and RDH expression, and decreased ROT-mediated ER stress-related gene expression in RPE cells.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

YH and RZ designed the research study. ZJ performed the research. ZQ provided help and advice on the methods of researchss. YR and YL analyzed the data. RZ and YH wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study applied the same PRE cell lines as our published studies [42]. The study was carried out in accordance with the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of The Second Affiliated Hospital of Xi'an Medical University (No. XZY202324).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2811319.

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