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Human glial müller and umbilical vein endothelial cell coculture as an in vitro model to investigate retinal oxidative damage. A morphological and molecular assessment

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Abstract

The aim of this study was to optimize a coculture in vitro model established between the human Müller glial cells and human umbilical vein endothelial cells, mimicking the inner blood-retinal barrier, and to explore its resistance to damage induced by oxidative stress. A spontaneously immortalized human Müller cell line MIO-M1 and human umbilical vein endothelial cells (HUVEC) were plated together at a density ratio 1:1 and maintained up to the 8th passage (p8). The MIO-M1/HUVECs p1 through p8 were treated with increasing concentrations (range 200–800 μM) of H_2O_2 to evaluate oxidative stress induced damage and comparing data with single cell cultures. The following features were assayed p1 through p8: doubling time maintenance, cell viability using MTS assay, ultrastructure of cell–cell contacts, immunofluorescence for Vimentin and GFAP, molecular biology (q-PCR) for GFAP and CD31 mRNA. MIO-M1/HUVECs cocultures maintained distinct cell cytotype up to p8 as shown by flow cytometry analysis, without evidence of cross activation, displaying cell–cell tight junctions mimicking those found in human retina, only acquiring a slight resistance to oxidative stress induction over the passages. This MIO-M1/HUVECs coculture represents a simple, reproducible and affordable model for in vitro studies on oxidative stress-induced retinal damages.

KEYWORDS
coculture, GFAP, HUVECs, MIO-M1 cells, oxidative stress

Research Highlights

- This direct glial/endothelial cell model might find a broad range of applications in retinal studies.
- The long-term availability, the chance to store cryopreserved stocks of each culture ready to use, and the affordable cost in the practice are strength points of this in vitro model.

Pasquinelli and Versura are joint senior authors.

1 | INTRODUCTION

The Müller cells (MCs) represent the principal glial population in the retina, crossing the structure from the inner to the outer limiting membrane, interacting with almost all retinal cell types (Coorey et al., 2012), and surrounding the blood vessels (Arroyo et al., 1997; Cunha-Vaz et al., 1966; Tout et al., 1993). MCs can be involved in several pathological conditions such as epiretinal membranes, macular holes and neovascularization related to ischemic retinopathies (Adamis et al., 1994; D'Amore, 1994; Hiscott et al., 1984), and are involved in neuroinflammation in glaucoma with progressive changes in morphology and function (Quaranta et al., 2021). The retinal damage activates MCs in order to protect the retinal tissue, undergoing morphological and functional alterations, like the up-regulation of the glial fibrillary acidic protein (GFAP) (Graca et al., 2018; Hippert et al., 2015). Due to their strategic localization and their multiple connections with almost all the retinal cell populations, MCs might represent the ideal target for the study of retinal diseases, including drug testing and neuroprotection strategies. Research in neurodegenerative disorders was first focused onto neuronal cell dysfunction, but the emerging role of glial cells in maintaining homeostasis, protecting neurons, and triggering the pathogenesis of the major neurodegenerative diseases has been also highlighted (Rõlova et al., 2021). Furthermore, the connecting role of endothelial cells and pericytes in the creation and maintenance of blood–brain barrier, and their cell–cell interaction, are also a key issue to be evaluated (Obermeier et al., 2016).

Studies performed on in vitro models from primary endothelial and glial cells in adult pig brain, showed that these cocultures maintained their properties for up to 8 weeks (Tanti et al., 2019), allowing to deepen the respective cell functions. A coculture cell model mirrors the in vivo context of the human tissue organization in a more reliable and accurate manner, than the single cell culture does.

To our knowledge, few studies have addressed this kind of culture, exploring direct physical contacts between retinal glial Muller and endothelial cells, of human origin. Due to difficulties in obtaining human primary cells from the retina, only mixed species in vitro model and one human multi-culture system have been previously published. A study by Yafai et al, performed an indirect coculture between bovine retinal endothelial cells and MIO-M1, where cells had faced each other but without any physical contact (Yafai* et al., 2004). Cocultures consisting of brain glia and endothelial primary cells of animal origin (Hornof et al., 2005; Lee & Tansey, 2013) have been proposed but with criticism related to the different species utilized. Most of the coculture models are in fact composed of mixed species or composed of cell lines and human or animal primary cells (Churm et al., 2019; Gu et al., 2003; Wisniewska-Kruk et al., 2012). A full human triple culture system of MIO-M1, Retinal Pigment Epithelial Cells (ARPE-19) and Primary Human Retinal Microvascular Endothelial Cells (HRMVEC) was recently proposed by using polyester Transwell membrane, with a complex, time consuming protocol, and only short period to use the model for experiments (Churm et al., 2019).

The purpose of the present study was to optimize a handling coculture cell model between human MIO-M1 (Moorfields/Institute of Ophthalmology-Müller 1) and human umbilical vein endothelial cells (HUVEC), by growing them in direct contact. The main goal of this approach is to provide a morphological characterization of this MIO-M1/HUVEC coculture system, and to explore the resistance to damage induced by oxidative stress.

2 | METHODS

2.1 | Cell culture

A spontaneously immortalized human Müller cell line MIO-M1 was obtained from the UCL Institute of Ophthalmology, London, UK (Limb et al., 2002). According to the manufacturer's instructions, MIO-M1 were grown in Dulbecco's Modified Eagle's medium with high glucose and Glutamax-I (Gibco, Life Technologies Ltd, Paisley, PA4 9RF, UK) supplemented with 10% Fetal Bovine Serum (Gibco, ThermoFisher Scientific 168 Third Avenue Waltham, MA USA) and 1% penicillin (10,000 U/ml)/streptomycin (10,000 µg/ml, Lonza Group Ltd., Basel, Switzerland). Cells were grown in culture flasks at 37°C in a humidified atmosphere containing 5% CO_2 and the media was changed every 2–3 days.

Human Umbilical Vein Endothelial Cells (HUVEC; Lonza Group Ltd., Basel, Switzerland) were cultured in the same media and conditions of MIO-M1 cell line, maintained at 37°C at 5% CO_2 and media was changed every 2–3 days until reaching the 80% cell confluency. Morphological analysis and the percentage of confluence of cell cultured was monitored every other day until confluence by inverted optical microscope.

2.2 | Coculture formation

The full contact coculture method in which cells are in complete physical contact was used to study the cross biological activity of MIO-M1 and HUVEC, and to check the cell–cell contacts. MIO-M1 at passage 56 and HUVEC at passage 38 were cultured according to the standard culture procedures. After 2 weeks, the single cell cultures were detached from the flask substrate with Trypsin – EDTA 1X in PBS solution (EuroClone, Milan, Italy), counted and plated together at a density ratio 1:1 under standard conditions (37°C, 5% CO_2). The MIO- M1/HUVEC coculture was subjected to a broad range of analysis for phenotype characterization, viability assay, and gene expression analysis. The preparation of the first MIO-M1 / HUVEC coculture was named p0. When confluence was reached, the cells were split and the first passage (named p1) was performed, proceeding in the same way until passage 8 (p8).

2.3 | Cell proliferation assessment

Cell growth was determined by plating 2.5 x 10^5 of both cytotypes into culture flasks and culturing in growth media. Routine cell passaging was performed every 4 days, cultured cells were collected by trypsin/EDTA treatment and counted with a counting chamber and replated at the same density. The process was repeated until p8. The doubling time (DT) was calculated as indicated in Zaniboni et al

(Zaniboni et al., 2014). Briefly, DT = h / CD, where h was the culture time in hours between two passages and cell doubling (CD) calculated as CD = ($\log_{10}$ N − $\log_{10}$ N_0) / $\log_{10}$ 2, *N* is the number of cells at 80%–90% confluency and N_0 is the number of cells seeded.

2.4 | Cell viability assay

To evaluate the coculture behavior and maintenance over the passages, a cell viability assay was performed. Cells were plated at a density of 1 × 10^5 cells/well in triplicate, in a 96-well plate. At 24 h after seeding, the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay with conversion to a soluble formazan product was performed according to the manufacturer's instructions (Promega CellTiter 96 AQueous One Solution Cell Proliferation Assay). Absorbance was measured at 490 nm using the Multiskan SkyHigh (ThermoFisher Scientific) microplate reader. Analyses were executed in three independent experiments, with three experimental replicates for each experimental point.

TABLE 1 Primer sequences and probes used for gene expression analysis

Gene	Primer sequence/probe accession number
CD31	FWD CACAGATGAGAACCACGCCT REV: GGCCCCTCAGAAGACAACAT
GAPDH	FWD: AATGGGCAGCCGTTAGGAAA REV: AGGAGAAATCGGGCCAGCTA Probe: Hs02786624_g1
GFAP	Probe: Hs00909233_m1

Abbreviations: CD31, Cluster of differentiation; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein.

2.5 | Immunofluorescence

Immunofluorescence was performed to detect the expression of GFAP in MIO-M1/HUVEC coculture. To this aim, 3 x 10^4 total cells (MIO-M1 + HUVEC) were seeded on glass slides. After 72 h, cells were washed with PBS and fixed/permeabilized with ice-cold methanol for 10 min at room temperature (RT). Incubation with 1% bovine serum albumin (BSA) in PBS, was performed to reduce non-specific binding for 30 min at RT. Slides were labeled with monoclonal primary antibody in 1% BSA/PBS for 1 h at 37 °C in a wet chamber (GFAP 1:500, Synaptic System). After primary antibody incubation, slides were washed with PBS and stained with anti-mouse Alexa Fluor 488 secondary antibody (1: 250, Life Technologies) for 1 h at 37 °C in the dark, and counterstained with ProLong Gold Antifade reagent with DAPI (Life Technologies). Finally, slides were observed in a Leica DMI6000 B inverted fluorescence microscope (Leica Micro-systems, Wetzlar, Germany).

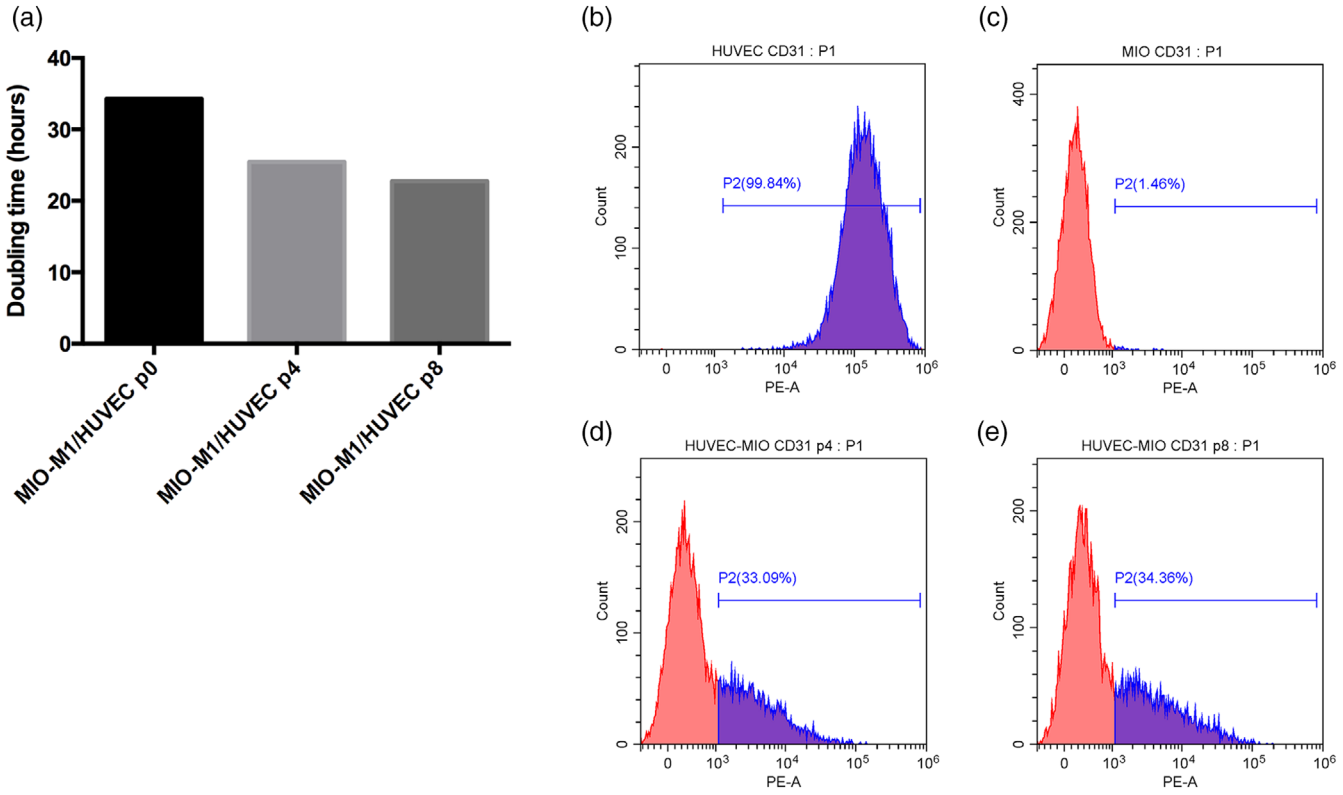

FIGURE 1 Characteristics of MIO-M1/HUVEC coculture. (a) Doubling time (DT) of MIO-M1/HUVEC coculture at p0, p4 and p8. (b) Analysis of CD31 expression by flow cytometer in HUVEC and (c) MIO-M1 as single cells and cocultures taken at (d) p4 and (e) p8. The percentage of CD31 positive cells was comparable between cocultures at p4 (33.09%) and p8 (34.36%)

2.6 | Flow cytometry

Immunophenotyping of HUVEC and MIO-M1 cells as well as MIO-M1/HUVEC co-cultures taken at P4 and P8 was analyzed using a flow cytometric approach. For staining each sample, 3 x 10^5 HUVEC or MIO-M1 cells and 5 x 10^5 MIO-M1/HUVEC coculture cells were collected in tubes, centrifuged at 300 x g for 5 min and washed three times in PBS. Cell pellets were resuspended in 100 µl 1% PBS/BSA for 15 min at RT for blocking unspecific staining before the addition of conjugated CD31 primary monoclonal antibody (human CD31/PECAM-1 PE-conjugated Antibody; 5–10 µl / 10^6 cells; R&D Systems, Inc. a Bio-Techne Brand), vortexed and incubated for 30 min at RT in the dark. Negative control was obtained omitting the primary antibody. After several washes with PBS, cells were resuspended in 500 µl of PBS and analyzed in a CytoFLEX S flow cytometer (Beckman Coulter, Milano, Italy) using CytExpert software version 2.3.1.22 for data analysis and graphs.

2.7 | Transmission electron microscopy (TEM)

Electron microscopy was performed to examine the ultrastructural features of MIO-M1 and HUVEC both as single cultures and cocultures. 8 x 10^3 total cells (MIO-M1, HUVEC and MIO-M1/HUVEC coculture taken at p0 and p8) were seeded in 6-well culture plates and incubated in a humidified incubator at 37°C and 5% CO_2 for 72 h.

Cells were fixed in plate using a 2.5% buffered glutaraldehyde solution for 20 min at RT. Then, each sample was scraped, harvested, and centrifuged at 300 x g for 5 min; the obtained cell pellets were stored at +4°C overnight in the same fixing solution. After that, the cells were rinsed in phosphate buffer, subjected to a post-fixation in 1% buffered osmium tetroxide for 1 h at +4°C, washed and dehydrated using ethanol at increased concentrations before embedding them in Araldite resin. Ultrathin sections were counterstained with uranyl acetate and lead citrate and observed in a Philips CM100 (FEI Company, ThermoFisher, Waltham, MA, USA) Transmission Electron Microscope. Images were taken with an Olympus Megaview II digital camera integrated with iTEM image processing software.

2.8 | Oxidative stress induction

Hydrogen peroxide (H_2O_2) at 30% (Sigma Aldrich) was used to induce oxidative stress. H_2O_2 was diluted in sterilized water at 9.88 mM, before preparation of working solutions in serum-free medium for cell treatments. MIO-M1 and HUVEC were seeded at a density of 1 x 10^4 cells/well in a 96-well plate for cell viability analysis and at a density of 1 x 10^5 cells/well in a 24-well plate and exposed to H_2O_2 in DMEM

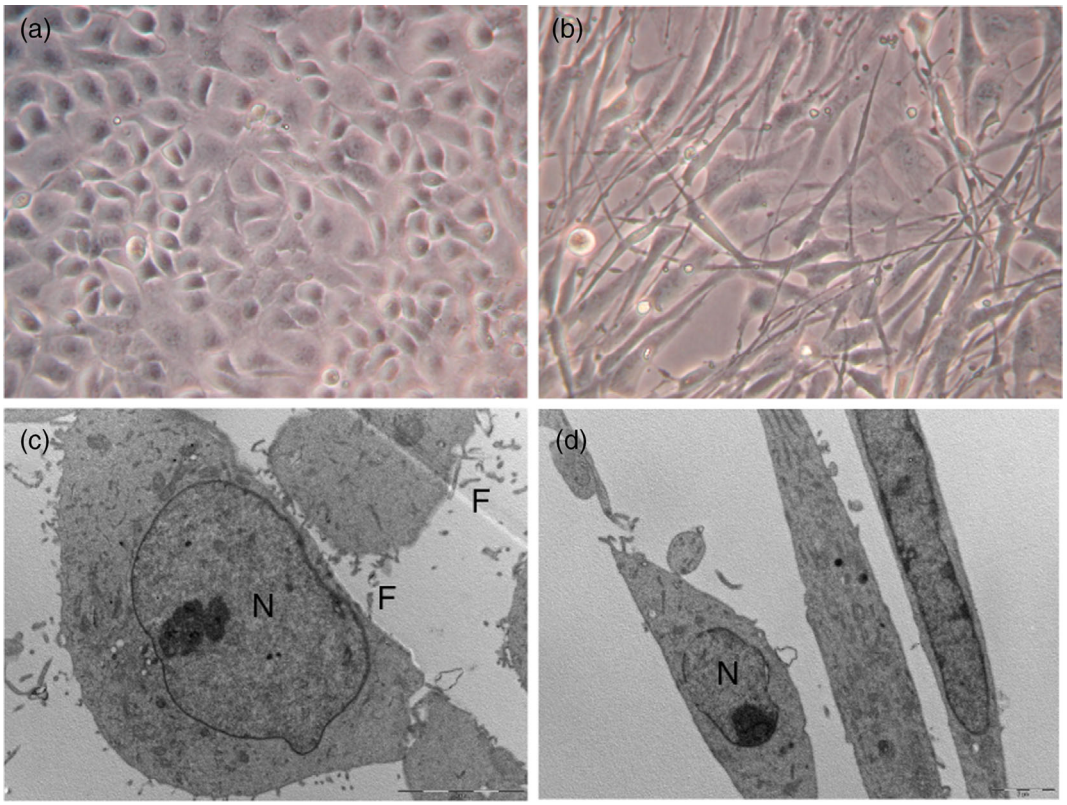

FIGURE 2 Morphological aspect of HUVECs and MIO-M1 cells. Representative images in inverted light and transmission electron microscopy of single HUVEC with the typical cobblestone-like morphology (a and c) and single MIO-M1 characterized by elongated shape (b and d). F: Filopodia; N: Nucleus. A and b: Images were captured at 20x of magnification. Scale bars: c = 5 µm; d = 2 µm

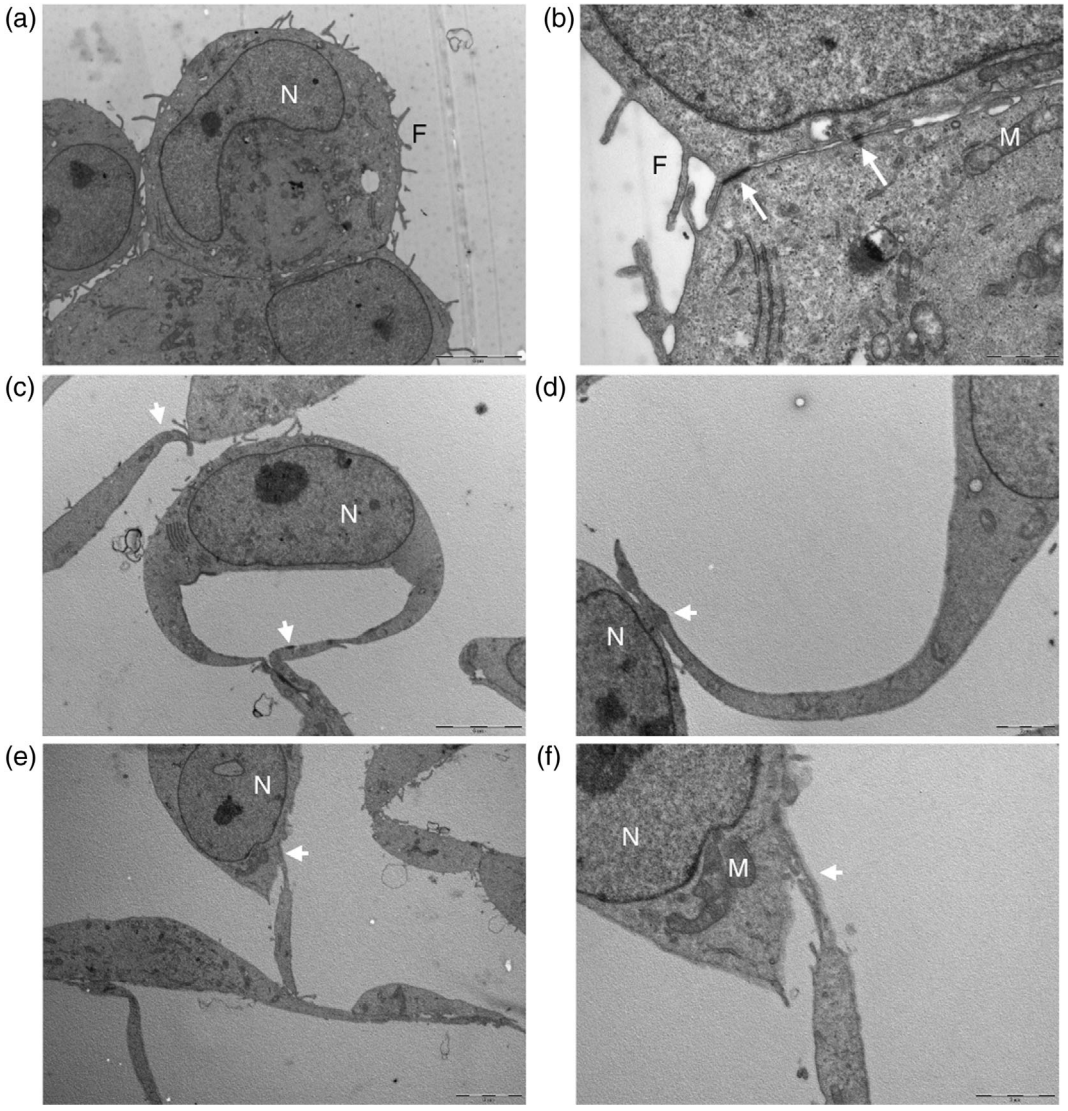

FIGURE 3 Ultrastructural features of MIO-M1/HUVEC in co-culture until p8. Representative TEM images of (a) a small cluster of HUVEC cells connected through (b) tight junctions (arrows). (c) Hook-like projections (arrowheads) of MIO-M1 used (d-e) to contact HUVEC cells (arrowheads). (f) A detail on the modality of interaction between MIO-M1 and HUVEC cells. F: Filopodia; N: Nucleus; M: Mitochondria. Scale bars: A, c and e = 5 μm; b = 1 μm; d and f = 2 μm

without serum at 200, 500 and 800 μM for 24 h at 37°C. Cells grown in DMEM without serum were used as controls.

The effect of H_2O_2 on MIO-M1/HUVEC coculture was evaluated in terms of cell survival. To this aim, MTS assay was performed, after 24 h exposure to oxidative stress, as above described. Analyses were executed in three independent experiments, with three experimental replicates for each experimental point.

2.9 | Gene expression analysis

HUVEC and MIO-M1 both as single cultures and in coculture at point p4 and p8 under normal conditions and after exposure to oxidative stress, were processed for gene expression analysis of CD31 and GFAP. Total RNA extraction was performed by using TRIreagent (TRIzol reagent, ThermoFisher Scientific) according to the manufacturer's instructions. RNA concentration was evaluated by QUBIT RNA Broad Range (Life Technologies) and one μg of total RNA was reverse transcribed in a 20 μl reaction volume using the iScriptTM cDNA synthesis kit (BioRad Laboratories) following the manufacturer's instructions. Real Time PCR analysis was carried on a CFX-96TM Real Time Detection System (BioRad Laboratories), using the semi-quantitative Sybr Green (Sso AdvancedTM Universal Sybr Green Supermix; BioRad Laboratories) and TaqMan (TaqMan Master Mix, Life Technologies) approaches. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Primer pairs were designed by using the NCBI Blast Tool (Sigma Aldrich, Milan, Italy) (Table 1). The probes used for TaqMan assays were: Human GFAP

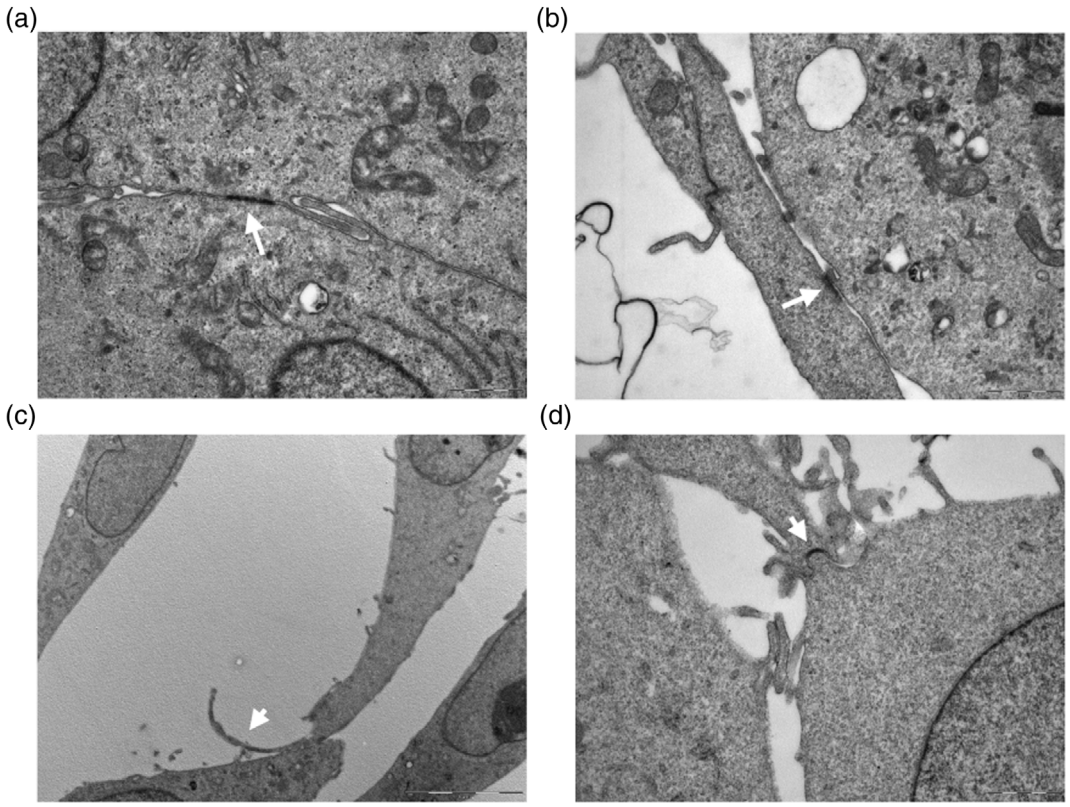

FIGURE 4 Intercellular connections in MIO-M1/HUVEC co-culture. Representative TEM images of (a) tight junctions (arrow) in HUVEC cells, (b) asymmetrical subplasmalemmal linear density (arrow) in MIO-M1/HUVEC co-culture (c) hook-like projections (arrowhead) and (d) button-type junctions (arrowhead). Scale bars: A, b and d = 1 μm; c = 5 μm

Hs00909233_m1; Human GAPDH Hs02786624_g1 (Life Technologies) (Table 1).

2.10 | Statistical analysis

Each experiment was executed at least in triplicate and analyzed through GraphPad Prism for graph elaboration and statistical analysis. Differences were considered statistically significant if the p value was lower than 0.05. Statistical analysis was performed through unpaired Student's t test for comparison between two groups and ordinary one-way Anova for comparisons among more than two groups.

3 | RESULTS

Characteristics of MIO-M1/HUVEC coculture - MIO-M1/HUVEC coculture was obtained by mixing the two cell populations at a density ratio of 1:1. The analysis performed at p0, p4 and p8 revealed a slight decrease of doubling time (Figure 1a). CD31 marker was used as a discriminating factor between MIO-M1 and HUVEC in flow cytometry analysis. As shown in Figure 1, CD31 was positive in HUVEC confirming the endothelial phenotype (Figure 1b), whereas it was absent in MIO-M1 (Figure 1c). In HUVEC/MIO-M1 cells coculture, the percentage of HUVEC cells positive cells was 33.09; similar value was observed also at p8 (34.36%) (Figure 1d-e).

Light microscopy and TEM were assessed to analyze the two cell populations along the culture passages. HUVEC cells showed a cobblestone-like appearance (Figure 2a), while MIO-M1 exhibited a fibroblast-like morphology with long cytoplasmic projections (Figure 2b). At ultrastructural examination, HUVEC cells showed an epithelioid morphology with perinuclear Golgi complexes, endocytic vesicles, tight junctions, and surface filopodia (Figure 2c), while the MIO-M1 cells were elongated with the cytoplasm rich in organelles, i.e., mitochondria, rough endoplasmic reticulum cisternae (Figure 2d); subplasmalemmal linear intercellular densities were also found. When cocultured until p8, the cells were integrated and less differentiated with poor organelle content (Figure 3a-f); as a remarkable finding, MIO-M1 displayed hook-like projections through which they made close connections with the HUVECs (Figure 3c-d and Figure 4c); these intercellular connections were strengthened by button-type junctions (Figure 4d). Tight junctions in HUVEC cells (Figure 4a) and asymmetrical subplasmalemmal linear density in MIO-M1/HUVEC coculture (Figure 4b) were also shown.

GFAP expression in MIO-M1/HUVEC coculture system - We analyzed the expression of GFAP in HUVEC and MIO-M1 both as single and dual cultures, to distinguish between the two cell populations

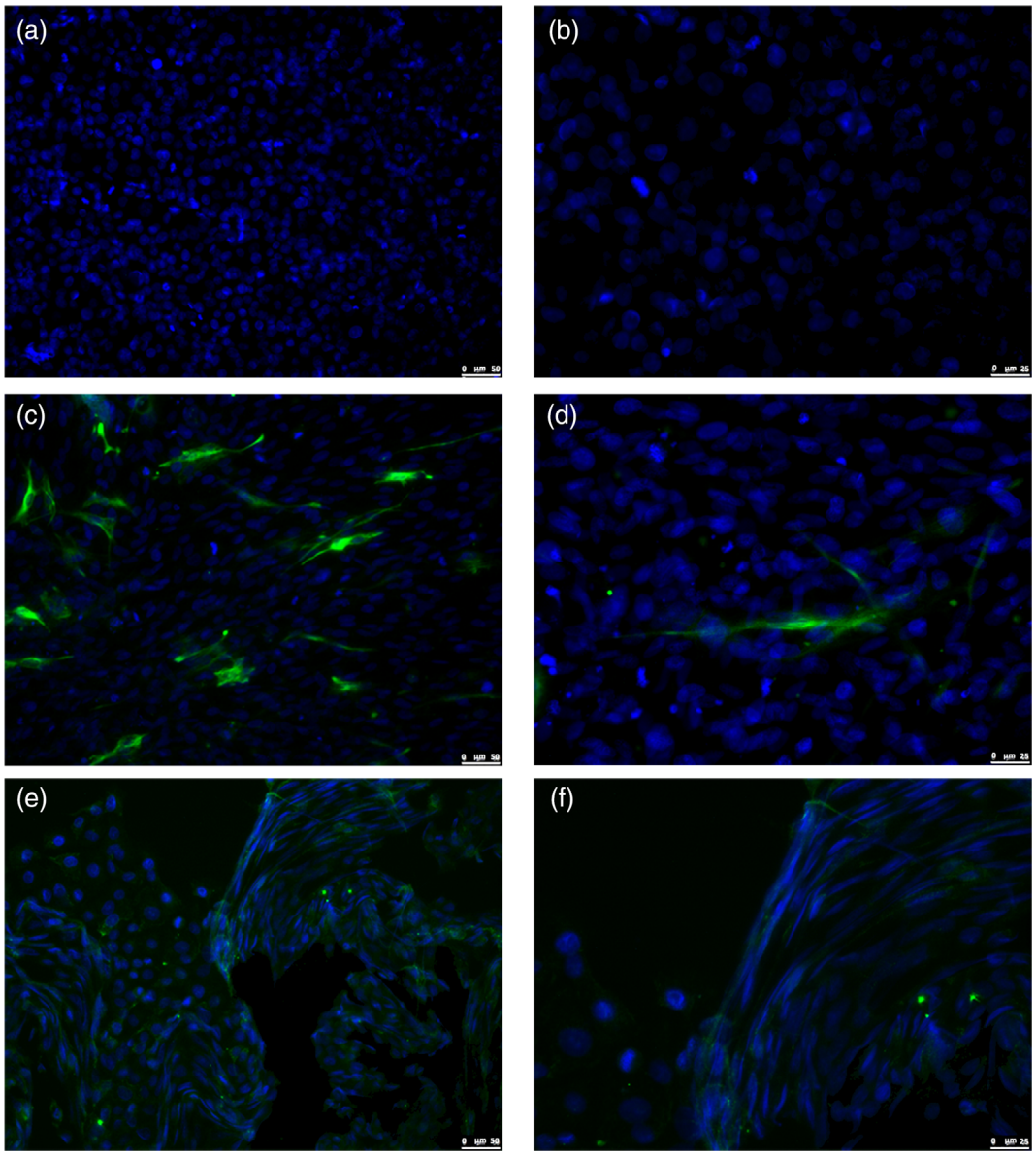

FIGURE 5 GFAP expression in MIO-M1/HUVEC coculture. Representative images of GFAP expression performed by immunofluorescence on (a-b) single HUVEC, (c-d) single MIO-M1, (e-f) MIO-M1/HUVEC coculture at p8. A, c, e: Images were captured at 20x of magnification and scale bars = 50 μm; b, d, f: Images were captured at 40x of magnification and scale bars = 25 μm. Nuclei: Blue fluorescence, GFAP positive cells: Green fluorescence. GFAP protein displayed a cytoplasmic pattern and was detectable only in MIO-M1 under physiologic conditions and in MIO-M1/HUVEC coculture at p8, exhibiting a low positivity that suggests a low level of MIO-M1 activation

and to evaluate the activation state of MIO-M1 following coculture. As it can be observed from immunofluorescence in Figure 5, GFAP expression shows a cytoplasmic pattern, detectable only in MIO-M1 at low levels under normal conditions (Figure 5a-d). When HUVEC and MIO-M1 were mixed until passage 8, we found low levels of GFAP, suggesting a low activation state of MIO-M1 (Figure 5e-f). Interestingly, the cell–cell interactions could be appreciated, highlighting MIO-M1 surrounding HUVEC. This structural organization mirrors the retinal neurovascular unit (Meng et al., 2021).

CD31 and GFAP gene expression in MIO-M1/HUVEC coculture - A molecular signature of MIO-M1/HUVEC coculture at p4 and p8 was explored. The expression of the endothelial cell marker CD31 was down-regulated in cocultures, when compared to single HUVEC cells. CD31 was reduced by 50% and 90% in p4 and p8 coculture, respectively (Figure 6a). The decrease in MIO-M1/HUVEC p4 can be explained with the mixed RNA belonging to the two distinct populations, considering that MIO-M1 cells do not express CD31. However, keeping the coculture until p8 determined a partial loss of CD31, suggesting the progressive loss of the endothelial cell lineage if the coculture is further prolonged beyond p8.

Further, we investigated the expression of GFAP, associated with retinal glial activation following damage or trauma. As shown in

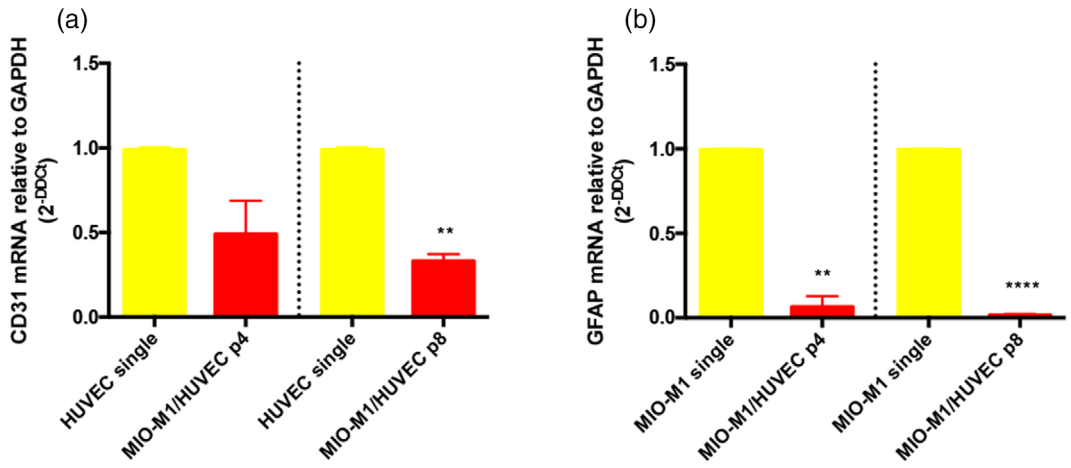

FIGURE 6 Gene expression analysis of MIO-M1/HUVEC co–culture. Transcriptional levels of (a) CD31 and (b) GFAP genes were analyzed in MIO-M1/HUVEC coculture at p4 and p8 by real time PCR. Transcript analysis revealed a decreasing trend for CD31 in MIO-M1/HUVEC coculture, more evident at p8, and low expression of GFAP, suggesting a poor activation of MIO-M1 when co-cultured with HUVEC. Statistical analysis was performed through unpaired *t* test; **, $p < .01$; ****, $p < .0001$

Figure 6b, a significant 90% down-regulation of GFAP occurred both at p4 and p8. This significant reduction excludes activation of MIO-M1 following the coculture with HUVEC, and as consequence the induction of a damage that can affect cell viability and functionality.

MIO-M1/HUVEC coculture under oxidative stress - In order to evaluate whether the MIO-M1/HUVEC coculture model was affected by oxidative stress-induced damage, we exposed the coculture at different passages (p0, p4 and p8) to H_2O_2 for 24 h. As shown in Figure 7, the oxidative stress resulted more cytotoxic on cocultures at the first establishment, indeed we observed a dose dependent decrease of cell viability. This drastic effect was attenuated in MIO-M1/HUVEC cocultures kept until passages 4 and 8, suggesting that the combination of these two cell populations may promote a neuroprotective effect (Figure 7a-b). We next investigated the gene expression of CD31 and GFAP in MIO-M1/HUVEC coculture at p4 and p8 under oxidative stress conditions. Based on cell viability data and on our previous findings (Ciavarella et al., 2020), H_2O_2 was used at 500 μM for 24 h. We observed up-regulation of CD31 mRNA in MIO-M1/HUVEC coculture exposed to H_2O_2 in comparison to untreated controls (30-fold increase in MIO-M1/HUVEC p4, p 0.076; 100-fold increase in MIO-M1/HUVEC p8, $p = 0.033$, unpaired t test) (Figure 8a), suggesting a potential modulation of cell damage through the stimulation of the endothelial differentiation and neo-vascularization. However, the low expression of GFAP transcript indicated that this response was not accompanied by MIO-M1 activation and cell suffering (Figure 8b).

4 | DISCUSSION

Data from the present study demonstrate that spontaneously immortalized human MIO-M1 cells and HUVEC can be grown together in a simple, affordable, and reproducible way for up to 8 passages, while maintaining their specific phenotype and structural integrity. In addition, prolonged coculture was not a stressor for MIO-M1 cells that preserved their ability to interact with HUVEC through well-organized intercellular contacts, without the enhancement of activation markers. Finally, an overall cell resistance toward oxidative stress was observed starting at p4.

The present study was performed with the aim to optimize a handling coculture system between human retinal glial cells and human endothelial cells. We focused on Müller cells (MCs), a glial cell population that regulates the retinal homeostasis, by secreting neuroprotective factors to preserve neuron nourishment and survival (Shen et al., 2012). Thus, MCs are crucial players during retinal diseases, representing an attractive cell target for studies on pathogenetic mechanisms, neuroprotection, and drug testing. Spontaneously immortalized human Müller cell line, MIO-M1, was cultured at direct physical contact with a HUVEC population, to mimic the interactions between MCs and blood vessels in retinal tissue. We recognized that vascular endothelial cell heterogeneity in structure and function exists which makes retinal endothelial cells unique (Bharadwaj et al., 2013; Rymaszewski et al., 1992). However, HUVEC are widely utilized as cell model in retina research to investigate several aspects from angiogenesis (Chen et al., 2017; Motiejūnaitė & Kazlauskas, 2008), to degeneration (Lidgerwood et al., 2019; Wareham & Calkins, 2020). In addition, HUVEC are a well characterized cell model, sharing their marker expression with the retinal microvascular endothelial cells (CD31/PECAM1, factor VIII, tubulogenesis) (Dye et al., 2004; Hewett & Murray, 1993), and sensitivity to VEGF and other growth factors (Eyre et al., 2020). Furthermore, commercially available retinal endothelial cells maintain their phenotype for a few passages only, whereas HUVEC appear more appropriate for models established for long term studies. Of course, primary cell sources might be closer to the native human retina, but extremely difficult to obtain. The tendency of primary cells to de-differentiate might yield to limited use of

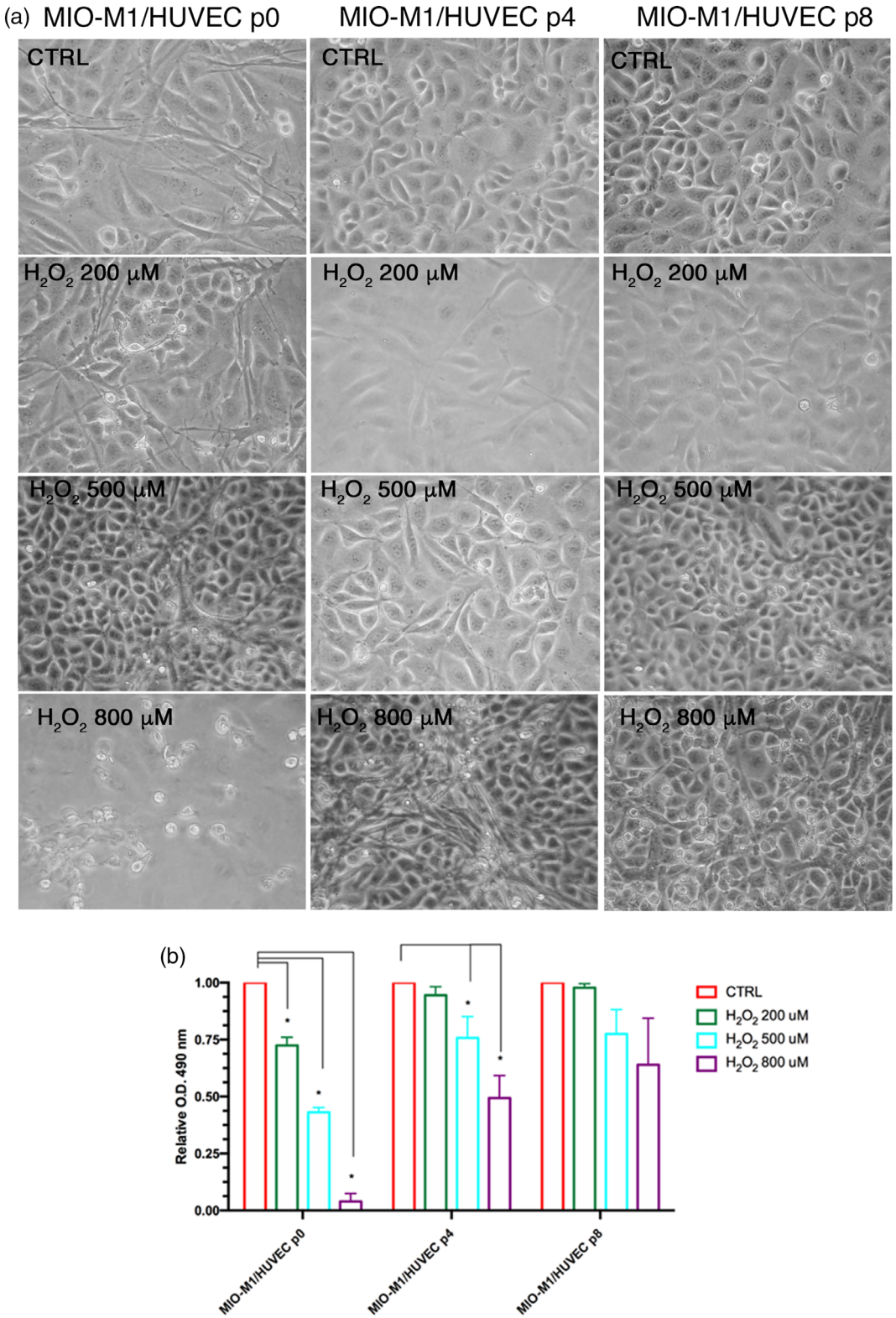

FIGURE 7 Effects of oxidative stress in MIO-M1/HUVEC coculture at p0, p4 and p8. (a) Morphological features of MIO-M1/HUVEC coculture exposed to H_2O_2 at 200–500-800 μM. (b) Cell viability of MIO-M1/HUVEC coculture measured through MTS assay. Data obtained suggested that the prolonged coculture of MIO-M1 and HUVEC ameliorates cell sensitivity to oxidative stress. Images were taken at 10x and 20x of magnification. Statistical analysis was performed through multiple t test; *, $p < .05$

passages, manipulation of culture media to mimic a disease effect, and minimized use in pharmacological testing. Primary human retinal microvascular endothelial cells and human retinal pericyte cell coculture studied by Eyre (Eyre et al., 2020) maintained their phenotype for 3 weeks. So, we proposed in this study the compromise of two highly characterized and routinely used cell lines.

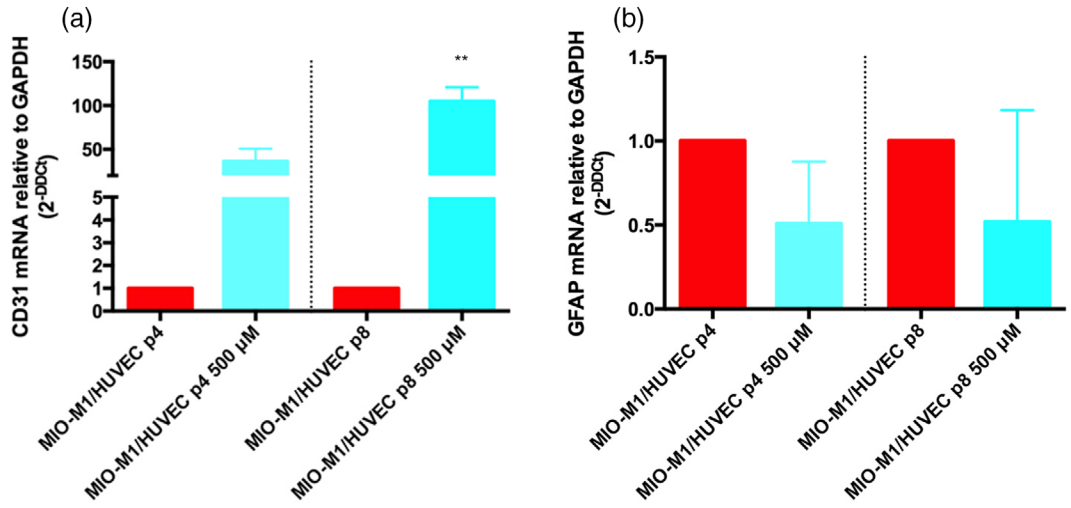

FIGURE 8 Gene expression analysis of MIO-M1-HUVEC coculture under oxidative stress. Transcriptional levels of (a) CD31 and (b) GFAP mRNA were performed in MIO-M1/HUVEC coculture at p4 and p8 exposed to H_2O_2 500 µM by real time PCR. The exposure to oxidative stress stimulated the expression of CD31, as a potential mechanism to manage stress. Statistical analysis was performed through unpaired t test; **, $p < .01$

GFAP expression in MCs is an indicator of tissue stress, and it has been associated with retinal degeneration. GFAP is a marker used to follow MCs behavior and response to any insult in in vitro model. Our model maintains MCs in their homeostatic conditions, without increased GFAP expression as a consequence of coculture with another cell phenotype. This is of value when considering the application of the model for neurodegenerative experiments.

In this respect, our coculture model was proved to be appropriate for investigations focused on oxidative stress induction. Interestingly, we found that the coculture system at late passages improved cell survival in comparison to the first one (p0), suggesting improved resistance of both cell populations when the coculture is prolonged over time. Further, an increase of angiogenic induction was observed under oxidative stress, suggesting the ability of HUVEC to survive and manage damage induction by upregulating the expression of CD31, an endothelial cell junction molecule involved in several biological processes like angiogenesis. In addition, we did not detect significant variations of GFAP mRNA in MIO-M1/HUVEC p4 exposed to oxidative stress, whereas a slight increase was found in MIO-M1/HUVEC coculture p8. Our data might suggest that the direct culture between MIO-M1 and HUVEC stimulates the gain of a more protective/resistance phenotype, where MIO-M1 cells are less susceptible to gliotic activation, even though cell morphology is maintained. Thus, experimental settings might be optimized for studying neuroprotection mechanisms in this coculture system at late passages.

In our study, human MIO-M1 and HUVEC were tightly connected, as shown by the ultrastructural demonstration of elaborated intercellular connections between MIO-M1 and HUVEC cells; in particular, MIO-M1 developed long cell projections terminated with a hook-like ending, resembling features occurring in the inner blood-retinal barrier in vivo (Dejana et al., 2009). These intercellular connections were strengthened by button-type junctions, which are believed to be specialized signaling complexes in endothelial cells (Dejana et al., 2009). A "full contact method" was described by Efremova et al., with murine astrocytes cultured in complete physical contact with human neurons (Efremova et al., 2017). This strategy reproduces the in vivo conditions, because mammalian tissues and other eukaryotic cell cultures often require direct contact to maintain physiological behavior (Bidarra et al., 2011; Méhes et al., 2012; van der Meer et al., 2013). For this reason, coculture systems have been used for cell–cell interaction studies, as well as becoming effective tools for evaluating neuroprotection. A direct coculture system represents a simple and practical strategy in the regenerative medicine and tissue engineering field to investigate the effects of soluble factors, like growth factors, small molecules, and cytokines, on cell synergy and behaviors on both cell populations (Paschos et al., 2015). Several studies have shown that monocultures of astrocytes and cocultures of these cells with neurons can detect neuroprotection effects (Saeed, Rehman, et al., 2015; Saeed, Xie, et al., 2015; Morken et al., 2005; Yin et al., 2009) based on cell type. Cells composing the coculture are able to monitor and actively respond to their requirement by conditioning the proliferation, organization, secretion of growth factors and cytokines, thus constituting a controlled environment that includes mixed-population monolayers on slides, flasks or dishes (Nishiofuku et al., 2011; Sugiyama et al., 2007) .

Alternatively, cells can be cultured together without being at physical contact, and can be separated by a Transwell system, consisting of a membrane insert that allows culturing different cell populations on different layers. At this regard, a study showed the neuroprotective effect of astrocytes on neurons, through an indirect coculture system where cells were physically separated by a transwell filter (De Simone et al., 2017). In further research, transwell

was used for co-culturing photoreceptor and Muller cells focusing on the effect of the supernatant of this system on microglia (Lu et al., 2017).

There are published in vitro coculture models that involve the development of 3D systems using scaffolds or gel matrices (East et al., 2009; Phillips & Otteson, 2011) .

In conclusion, the present study proposes a direct glial/endothelial cell model, which might find a broad range of applications in the field of retinal biology and disease pathogenesis, in particular focusing on oxidative stress induced damages. Strength points of our coculture model are the long-term availability of the model, the chance to store cryopreserved stocks of each culture ready to use, and the affordable cost in the practice.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study did not require ethical approval.

INFORMED CONSENT STATEMENT

Not applicable.

AUTHOR CONTRIBUTIONS

Gloria Astolfi: Data curation; investigation; methodology; validation; writing – original draft; writing – review and editing. **Carmen Ciavarella:** Investigation; methodology; validation; writing – original draft. **Sabrina Valente:** Data curation; investigation; methodology; validation; writing – original draft. **Chiara Coslovi:** Data curation; investigation; methodology. **Danilo Iannetta:** Data curation; methodology; writing – original draft. **Luigi Fontana:** Conceptualization; methodology; writing – review and editing. **Gianandrea Pasquinelli:** Conceptualization; investigation; methodology; writing – original draft. **Piera Versura:** Conceptualization; funding acquisition; methodology; project administration; supervision; validation; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funder (Fondazione Cassa di Risparmio Bologna, grant 2019.0547 to PV) had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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