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Acellular Amniotic Membrane Extract Reduces Oxidative Stress, Complement, and Angiogenic Pathways in Retinal Cells

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Abstract

Background: Oxidative stress (OS) and subsequent mitochondrial dysfunction represent critical early upstream events in the degeneration of human high demand metabolic tissues, such as those in the retina. Under persistent oxidative conditions, the accumulation of reactive oxygen species (ROS) triggers chronic inflammation. In retinal tissues, local complement system orchestrates inflammatory signal release and pro-angiogenic program activation leading to progressive cell failure and abnormal vessel growth. Given the limitations of single-target therapies, multi-target cytoprotective strategies are urgently required. While the human amniotic membrane (AM) exhibits well-established anti-inflammatory and tissue-repair properties and is broadly applied in ophthalmic pathologies, its underlying biochemical mechanisms remain poorly defined. This study investigated whether acellular AM-derived factors could protect human retinal pigment epithelium (RPE) cells, ARPE-19, against hydrogen peroxide-induced injury and modulate downstream complement, inflammatory, and angiogenic signaling.

Methods and results: ARPE-19 cells were exposed to H_2O_2 and treated with acellular AM extract in both pre-treatment and post-treatment protocols. Cell viability was assessed via MTT assay, and intracellular ROS accumulation was quantified. Mitochondrial network connectivity and morphological fragmentation were evaluated through quantitative skeletal imaging analysis. Monolayer integrity and cytoprotection were verified by wound-healing and cell attachment assays. Transcriptional profiles of local complement components (C3 and C5), complement receptors (C3aR and C5aR), pro-inflammatory cytokines (IL-6 and IL-8), and angiogenic factors (HIF-1α and VEGF) were quantified by RT-qPCR.

Our results shown that the exposure to H_2O_2 induced a significant, dose-dependent decrease in cell viability, which fell to 35% at 0.2 mM. AM pre-treatment robustly maintained cell viability above 85%, while post-treatment also offered significant rescue against oxidative damage. Both AM administration protocols successfully halted intracellular ROS accumulation and effectively prevented mitochondrial network breakdown, restoring the elongated, interconnected tubular morphology of active mitochondria. Furthermore, AM factors preserved monolayer integrity, preventing cell detachment and degeneration in wounded areas. At the molecular level, oxidative injury triggered a sharp upregulation of complement mediators (C3, C3aR, C5aR), pro-inflammatory cytokines (IL-6 and IL-8), and pro-angiogenic factors (HIF-1α and VEGF). Notably, both AM treatment regimens significantly suppressed these pathogenic pathways, with a particularly pronounced inhibitory effect on upstream angiogenic signaling (HIF-1α) and complement transcription.

Conclusion: These findings demonstrate that acellular AM-derived factors act as potent multi-target modulators that intercept oxidative stress, restore mitochondrial network connectivity, and suppress downstream complement-

driven inflammation and angiogenic signaling in an *in vitro* human cell model. This study provides crucial mechanistic evidence supporting the development of AM-based biochemical therapies for complex degenerative applications.

Keywords: Amniotic Membrane; ARPE-19 cells; Oxidative Stress; Mitochondrial Dysfunction; Complement System; Angiogenesis.

Abbreviations: AM: Amniotic Membrane; AMD: Age-related Macular Degeneration; C3: Complement component 3; C3aR: Complement component 3a Receptor; C5: Complement component 5; C5aR: Complement component 5a Receptor; Ctrl: Control; DCFH-DA: 2',7'-dichlorofluorescin diacetate; EGF: Epidermal Growth Factor; EMT: Epithelial-Mesenchymal Transition; FBS: Fetal Bovine Serum; FGF: Fibroblast Growth Factor; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; H_2O_2: Hydrogen Peroxide; HIF-1α: Hypoxia-Inducible Factor 1-alpha; IL-6: Interleukin 6; IL-8: Interleukin 8; LSP: Longest Shortest Path; OS: Oxidative Stress; PBS: Phosphate Buffered Saline; ROS: Reactive Oxygen Species; RPE: Retinal Pigment Epithelium; VEGF: Vascular Endothelial Growth Factor.

Introduction

Oxidative stress (OS) and subsequent mitochondrial dysfunction represent central features in the degeneration of human high-demand metabolic tissues. Cellular systems characterized by this condition are particularly susceptible to reactive oxygen species (ROS) accumulation, which progressively disrupts mitochondrial network integrity and accelerates cellular failure [1,2]. While endogenous antioxidant systems typically maintain redox homeostasis, prolonged exposure to environmental or physiological stressors disrupts this equilibrium. The resulting accumulation of ROS leads to OS, causing progressive cell damage and tissue degeneration [3]. In high-energy-demand tissues such as those of the retina, persistent OS triggers chronic inflammatory cascades, where the local complement system serves as a crucial upstream regulatory pathway [4,5]. Anaphylatoxins C3a and C5a, derived from the cleavage of complement factors C3 and C5, act through their specific receptors (C3aR and C5aR) to activate robust pro-inflammatory networks. Simultaneously, these cascades could drive to pro-angiogenic programs, through the induction of Vascular Endothelial Growth Factor (VEGF) production [6,7]. In addition, complement-driven signaling intersects with hypoxia-responsive pathways, orchestrating the upregulation of Hypoxia-Inducible Factor 1-alpha (HIF-1α) and the subsequent induction of HIF-1–dependent VEGF transcription [8]. This molecular convergence creates a self-perpetuating cycle of oxidative damage, complement activation, and pro-angiogenic signaling, central in retinal degenerative pathologies [9]. Given that conventional targeted therapies often fail due to compensatory escape mechanisms within these interconnected pathways, there is an urgent need to identify multi-target cytoprotective agents capable of simultaneously mitigating mitochondrial decay, inflammation, and angiogenic induction.

The Human Amniotic Membrane (AM) has long been used for a long time as a compelling biological source of complex biomolecules with potent anti-inflammatory, anti-scarring, and cytoprotective properties, while lacking immunogenicity and behaving as an inert scaffold. [10,11]. The AM features an intricate extracellular matrix and a rich reservoir of growth factors, with multiple studies describing its composition and characterizing the half-life of its distinct components [12,13]. Consequently, AM acts as a dynamic modulator of the cellular microenvironment rather than a passive support [14]. Recent biological evidence has highlighted the capacity of AM extracts to promote cell survival, support proliferation, and protect against oxidative damage [15]. Patients with retinal pathologies transplanted with AM showed improvements in cellular performance and tissue recovery [16]. Additionally, AM extracts promote cell survival and prevent epithelial-mesenchymal transition (EMT) induced by oxidative stress [17]. Notably, a recent study by Lulli et al. (2024) demonstrated that human amniotic membrane extracts exert profound survival effects on human ARPE-19 cells [17]. However, many of its properties

remain unexplored, and the precise molecular crosstalk through which acellular AM-derived factors intercept mitochondrial fragmentation, local complement amplification, and angiogenic responses remains to be fully elucidated. Given the wide spectrum of beneficial properties and the unlimited availability of AM, a better understanding of its effects would widen its therapeutic applications.

In this study, we hypothesize that acellular AM-derived factors can act as a multi-target mechanism to intercept metabolic failure and suppress concurrent pro-inflammatory and pro-angiogenic cascades in retinal pigment epithelium (RPE) cells under pathological conditions. Therefore, this work systematically analyzes the cytoprotective effects of AM extracts against hydrogen peroxide-induced oxidative injury using the human retinal-derived ARPE-19 cell line. We specifically investigate cell survival, intracellular ROS production, and mitochondrial network connectivity through quantitative skeletal imaging, while elucidating the transcriptional modulation of local anaphylatoxin ligands and receptors (C3, C5 and C3aR/C5aR), pro-inflammatory interleukins (IL-6/IL-8), and upstream angiogenic regulators (HIF-1α/VEGF) in this *in vitro* ocular model.

Materials and Methods

Processing of Amniotic Membrane (AM) extracts

Amniotic membrane extracts were provided by the Amnios Tissue Bank (https://www.amniosbma.org/), in strict accordance with the Declaration of Helsinki and with its adequate ethics approvals archived at the Tissue Bank. Each extract was derived from an individual AM, that was homogenized mechanically (ULTRA-TURRAX) and centrifuged (10,000 rpm, 4°C, 10 min). The resulting supernatant was filtered (0.2 µm), quantified for protein content using the Bradford assay, and stored at -80°C.

Cell viability assay

ARPE-19 cells (ATCC, Manassas, VA, USA) were cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS). To induce oxidative stress (OS), cells were seeded in 24-well plates (15,000 cells/cm^2) and exposed to H_2O_2 (concentration: 0.1, 0.2 and 0.3 mM) in DMEM-F12 with 1% FBS for 24 h. Control cells were kept in 1% FBS medium treated only with PBS (Control, Ctrl) or with AM (AM Control, AM Ctrl). To evaluate the protective effects of AM, two protocols were tested: pre-treatment (AM PRE, concentration of AM used based on protein concentration: 0.2 µg/µl), applied 3 h before H_2O_2 exposure, and post-treatment (AM POST, concentration of AM: 0.2 µg/µl), applied 3 h after H_2O_2 exposure (**Figure 1**). Cell viability was determined via the MTT assay and normalized against the control group. An IC 50% value of approximately 0.2 mM for H_2O_2 was established and utilized for all subsequent assays. Experiments were performed at least in triplicate.

Levels of Reactive Oxygen Species (ROS) production assay

Intracellular ROS levels were evaluated using the 2',7'-dichloroflourescin diacetate probe (DCFH-DA, Sigma). ARPE-19 cells were seeded on coverslips in 24-well plates and subjected to the Ctrl, PBS, AM PRE, and AM POST treatments using 0.2 mM H_2O_2. After 24 h, cells were incubated with 10 µM DCFH-DA in serum-free medium for 30 min at 37°C, washed three times with PBS, and imaged using a Leica DM2700 M fluorescence microscope equipped with a Leica DMC6200 camera. For fluorescence quantification, the same protocol was performed without coverslips; cells were detached with trypsin, transferred to 500 µL tubes, and read on a DeNovix QFX Fluorometer (blue channel: 495 nm excitation / 529 nm emission). Cells were subsequently lysed by three freeze-thaw cycles and centrifuged (10,000 rpm, 4 °C) to isolate the supernatant for protein quantification via the Lowry method.

Mitochondrial morphology analyses

Active mitochondria were labeled using MitoTracker Red 580 (Molecular Probes). Following the treatment conditions outlined for ROS evaluation, cells were incubated with 2 µM MitoTracker in serum-free DMEM-F12 for 40 min at 37 °C. Microphotographs were acquired using a Leica DM2700 M fluorescence microscope with a Leica DMC6200 camera. Mitochondrial network connectivity was quantified by measuring the Longest Shortest Path (LSP) using Fiji/ImageJ. At least six images from three independent experiments were analyzed per group.

Wound healing assay

ARPE-19 cells were grown to 90% confluence in 24-well plates, and a linear scratch was made using a sterile 100–1,000 µL pipette tip. Cellular debris was removed by washing with PBS. Cells were then exposed to 0.2 mM H_2O_2 and treated with PBS or AM (AM PRE and AM POST regimes, as described above). Control cells received PBS without H_2O_2. Baseline and 24 h post-scratch microphotographs were captured at identical regions using an inverted phase-contrast microscope (Olympus CKX41 SF, 40x magnification) for qualitative analysis.

RT-qPCR measure of inflammatory and angiogenic molecules

Total RNA was isolated from treated ARPE-19 cells in 6-well plates using 1,000 µL TRIreagent (Genbiotech, Buenos Aires, Argentina) following the manufacturer's protocol. RNA concentration and purity were assessed by A260:A280 spectrophotometry. Total RNA (3 µg) was reverse-transcribed into cDNA in a 20 µL reaction volume containing 50 pg OligodT primers (Biodynamics), 1x Superscript III reaction buffer, 0.5 mM dNTPs, 200 UI Superscript III Reverse Transcriptase (Invitrogen, USA), and 5 µM DTT. Quantitative PCR was performed in a 15 µL final volume using SSO Advanced Universal Mix SYBR Green (Bio-Rad Laboratories, USA) and 0.8 µM of specific primers. Primers for IL-6, IL-8, C3, C5, VEGF, HIF-1α, and GAPDH were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) or obtained from literature (Table 1) [18-21] while

pre-designed primers for C3aR and C5aR were sourced from Sino Biological (PR, China). Amplification was conducted on a StepOne Real-Time PCR System (Applied Biosystems) using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a melting curve analysis. Relative mRNA expression was calculated using the ΔΔCt method, with the Control group serving as the calibrator and GAPDH as the reference gene. Assays were repeated at least three times.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments. Statistical significance was evaluated by one-way ANOVA followed post-hoc tests (Newman-Keuls for no parametric analysis and Bonferroni for parametric). Values of $p < 0.05$ were considered statistically significant. Microscopic images are representative of three independent evaluations.

Results

Amniotic Membrane Preserves Viability and Attenuates Oxidative Stress

Exposure to H_2O_2 for 24 h induced a significant, dose-dependent decrease in ARPE-19 cell viability in the PBS group compared to the control (Ctrl), validating the oxidative stress (OS) model. Viability dropped to 35% at 0.2 mM H_2O_2, which was selected for subsequent assays. AM pre-treatment (AM PRE) was the most effective intervention, maintaining cell viability near or above 85% across all H_2O_2 concentrations (0.1–0.3 mM). AM post-treatment (AM POST) also offered significant protection at 0.2 mM H_2O_2 and 0.3 mM ($p < 0.01$ and 0.05 respectively). Baseline experiments showed that AM alone (AM Ctrl) had no effect on viability and was excluded from further analyses (**Figure 2**).

Regarding ROS production, DCFH-DA microphotographs and fluorometry revealed a marked increase in intracellular ROS levels in the H_2O_2 + PBS group compared to Ctrl. Both AM PRE and AM POST treatments successfully abated this accumulation, returning fluorescence intensity to near-control values (**Figure 3**).

Amniotic Membrane Protects Mitochondrial Morphology

MitoTracker Red staining revealed that Ctrl cells exhibited a well-defined, interconnected, and elongated filamentous mitochondrial network distributed throughout the cytoplasm. Conversely, the H_2O_2 + PBS group displayed a complete loss of network integrity, characterized by fragmented, punctate mitochondria with marked perinuclear clustering. Both AM PRE and AM POST treatments attenuated this fragmentation, noticeably restoring the elongated, tubular mitochondrial morphology (**Figure 4a**). Quantitative analysis via the Longest Shortest Path (LSP) metric validated these visual findings. The H_2O_2 + PBS group exhibited a significant reduction in LSP values compared to Ctrl, reflecting network breakdown. Notably, both AM treatment protocols effectively

prevented or reversed this reduction, yielding significantly higher LSP values that confirmed the preservation of mitochondrial connectivity (**Figure 4b**).

Amniotic Membrane Preserves Monolayer Integrity in Oxidative Stress conditions

We have analyzed the effect of AM treatments on monolayers of H_2O_2-exposed RPE cells, by wound assay.

We have observed that H_2O_2 addition follow PBS treatment induced degeneration and detachment of cells in the wound area, while AM treatment protected the monolayer. Pre-treatment induced preservation of the monolayer with some cell presents in the scratch area. Post-treatment retained the adherence of the monolayer in the scratch area, without signs of migration (**Figure 5**).

Amniotic Membrane Downregulates Anaphylatoxin Signaling in Oxidative Stress Conditions

To evaluate complement-mediated responses, mRNA levels of C3, C5, C3aR, and C5aR were quantified. Exposure to 0.2 mM H_2O_2 + PBS induced a sharp upregulation of both C3aR and C5aR transcripts compared to Ctrl. This complement receptor upregulation was effectively suppressed by both AM PRE and AM POST treatments, restoring expression to near-baseline levels. Regarding local complement components, C3 mRNA was constitutively expressed in Ctrl cells, whereas C5 mRNA was undetectable. OS exposure induced a significant two-fold increase in C3 expression, which was fully restored to control levels by both AM protocols (**Figure 6**.

Amniotic Membrane Modulates the Pro-inflammatory Cytokine Profile in Retinal Pigment Epithelium Cells Exposed to Oxidative Stress

Concomitant with the modulation of complement signaling, the expression of key inflammatory mediators was analyzed. Under OS (H_2O_2 + PBS), RPE cells exhibited a massive upregulation of IL-6 and IL-8 mRNA, indicating a shift toward a pro-inflammatory phenotype. Remarkably, both AM PRE and AM POST treatments robustly suppressed this cytokine spike, achieving a nearly complete rescue of the cells back to control expression profiles (**Figure 7**).

Amniotic Membrane Downregulates Angiogenic Factor production in Oxidative Stress conditions

To determine the impact of AM on neovascular signaling, HIF-1α and VEGF expression was assessed. Exposure to 0.2 mM H_2O_2 + PBS significantly upregulated both genes, favoring a pro-angiogenic microenvironment. Both AM treatment protocols effectively attenuated these increases. Notably, the inhibitory effect of AM was particularly pronounced on HIF-1α transcription, indicating that AM factors interfere with early-stage hypoxic/oxidative signaling upstream of pathological angiogenesis (**Figure 8**).

Discussion

This study shows that acellular AM-derived factors protect ARPE-19 cells under *in vitro* oxidative stress. AM improved cell viability, reduced ROS accumulation, preserved mitochondrial morphology and connectivity, and prevented oxidative stress–associated deterioration of RPE monolayer organization. Mechanistically, AM most strongly suppressed OS-induced complement-associated signaling, including complement receptors (C3aR and C5aR) and C3 expression, and it concurrently downregulated pro-inflammatory cytokines (IL-6 and IL-8) and pro-angiogenic factors (HIF-1α and VEGF).

As described, H_2O_2 induces RPE injury through ROS accumulation and mitochondrial fragmentation [22]. In our model, AM reduced the mitochondrial damage, probably contributing to limiting ROS increases, and interrupting the cycle that further amplifies OS and dysfunction. This is supported by prior reports that placenta-derived stem cells mitigate H_2O_2-driven mitochondrial injury [23]. In addition, AM contains growth factors (e.g., EGF, FGF) that can activate pro-survival signaling under OS [24,25]. Notably, our findings strongly align with and extend those of Lulli et al. (2024) and Krishna et al. (2021), who demonstrated that human amniotic membrane extracts exert profound survival effects on human ARPE-19 cells [17,15]. By elucidating the downstream molecular pathways, our data provide a mechanistic basis for these observed cytoprotective effects.

AM treatment also improved monolayer integrity and reduced OS-associated remodeling as it was also demonstrated [17]. In the wound-healing assay, AM applied after injury, limited both monolayer detachment and cell degeneration, suggesting that AM could counteract oxidative stress–triggered cell degenerative behavior and cell transformation [26].

Chronic, dysregulated inflammation is a key driver of retinal injury, and RPE cells contribute to a pro-inflammatory microenvironment during aging and AMD [27,28]. Complement signaling is an important upstream regulator in retinal degenerative pathologies such as age related macular degeneration [29], with C3 linked to late-stage progression and complement factor H polymorphisms associated with increased risk [30,31]. Consistently, stress enhances local C3 production in RPE and promotes both inflammatory and pro-angiogenic programs under oxidative stress [9,32]. In our model, H_2O_2 alone induced increases in complement receptor expression (C3aR, C5aR) and C3, whereas AM restored these markers toward control levels. By suppressing complement-driven amplification, AM plausibly explains the reductions in IL-6/IL-8 and in pro-angiogenic signaling observed in our study. Inhibitory drugs against the C3a component, has been tested to deal with AMD with promising results [33]. One crucial aspect in retinal pathologies is the new vessel growth, that principally obliterates the macular area and produces leaked fluids. The available therapies antagonize the VEGF availability, but they do not solve the production of this factor. It has been shown by other authors that AM reduces the levels of VEGF production by

OS injured RPE cells [15]. Similarly, our AM preparations reduced not only the VEGF increase, but also HIF-1α augmentation on OS *in vitro* affected RPE cells, highlighting the property of this material to suppress the pro-angiogenic effect of the hypoxic signals. This reduction could be linked to restored C3aR and C5aR levels, since C3a and C5a have been shown to regulate VEGF production in injured RPE cells [9]. Oxidative stress, coupled with hypoxic conditions, leads to an imbalance in lipid metabolism primarily linked to the C3aR/HIF-1α axis [8]. This suggests that HIF-1α dysregulation in RPE cells under OS condition, could not only trigger VEGF production, but also contributes to the functional impairment of these cells. The restoration of HIF-1α to control levels observed with AM treatments in our studies would indicate not only a reduction in angiogenic signaling but also an improvement in metabolic functioning of RPE cells under OS.

A central inflammatory molecule dysregulated in retinal pathologies is IL-6 which has been indicated as direct regulator of the production of VEGF [34]. Studies in patients with Diabetic Macular Edema show a direct positive correlation between the concentrations of IL-6 and VEGF in the vitreous humor, and patients who do not respond well to anti-VEGF drugs (such as ranibizumab) often have very high levels of IL-6 [35,36]. Our results showed that AM induced a profound downregulation of IL-6 in OS condition, also demonstrating the valuable activity of this material on a broad spectrum of pathological misbalanced cellular cascades, counteracting inflammation and altogether the angiogenic induction.

Conclusion

In conclusion, our results point to a unifying mechanism in which AM-derived factors act upstream of multiple pathogenic pathways. By preserving mitochondrial function, AM treatment may simultaneously reduce oxidative stress, dampen complement activation, and limit pro-angiogenic signaling. These results contribute to a better understanding of the mechanisms that could be acting on diverse application of this material and also could contribute to broaden its applications.

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Competing Interests

No potential conflict of interest was reported by the author(s).

Declarations

This study did not involve human participants, human data, or live vertebrate animals. All the amniotic membrane samples were obtained with the corresponding informed consent and approval of ethics committees of health centers and are storage at amniotic membrane bank (AMNIOSBMA tissue bank, https://www.amniosbma.org/).

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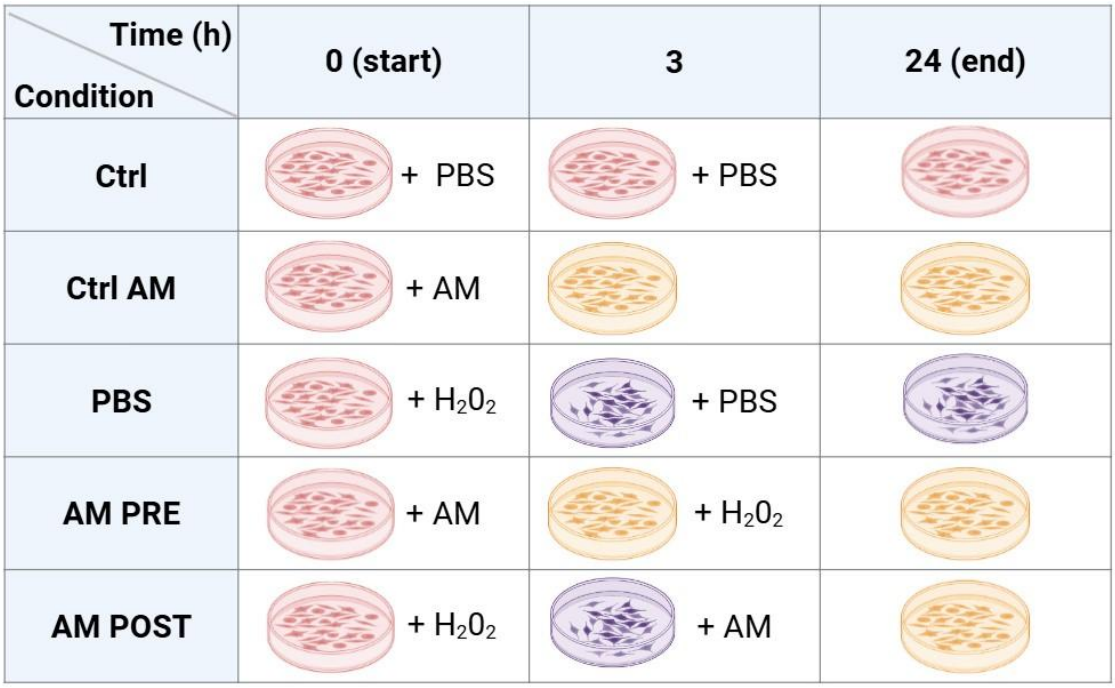
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Table 1: Primer sequences

Gene	Accession number	Forward Primer (5'→3')	Reverse Primer (5'→3')	Product (bp)
GAPDH	NM_002046.7	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA	81
C3	NM_000064.4	TTCCGATTGAGGATGGCTCG	ATGTCACTGCCTGAGTGCAA	141
HIF-1α	NM_001530.4	GTACCCTAACTAGCCGAGGAAGAA	GTGAATGTGGCCTGTGCAGT	79
VEGF	NM_001025366.3	GGGCAGAATCATCACGAAGT	TGGTGATGTTGGACTCCTCA	210
IL-6	NM_000600.4	GATGAGTACAAAAGTCCTGATCCA	CTGCAGCCACTGGTTCTGT	130
IL-8	NM_000584.3	TCTGTGTGAAGGTGCAGTTTT	GTGTGGTCCACTCTCAATCAC	127

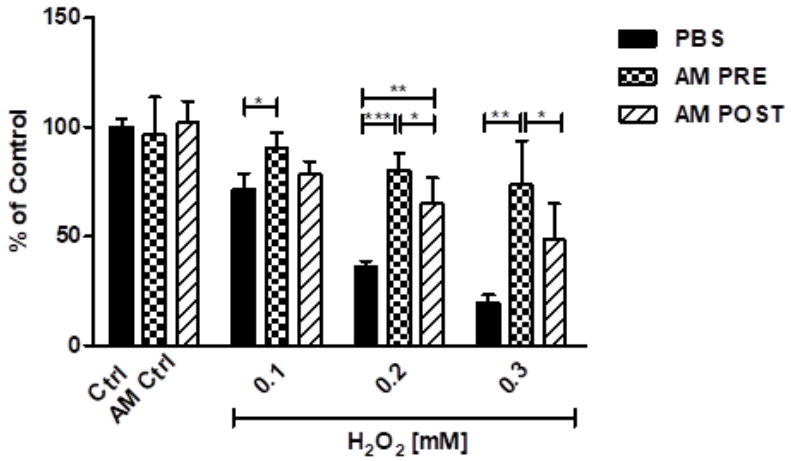
The primers were design by Blast primer-3 program or were obtained from previous published reports cited in the materials and methods section.

Fig. 1



Experimental design and treatment timeline for ARPE-19 cells. Experimental groups were Control (Ctrl), AM Control (AM Ctrl), H_2O_2 plus PBS (PBS), and H_2O_2 plus AM administered either 3 hours before (AM PRE) or 3 hours after (AM POST) exposure to the injury agent. The timeline shows the beginning of treatments (0, start), with second intervention after 3 h (3) and the final endpoint at 24 h (24, end). PBS: Phosphate Buffered Saline; H_2O_2: Hydrogen Peroxide; AM: Amniotic Membrane extract.

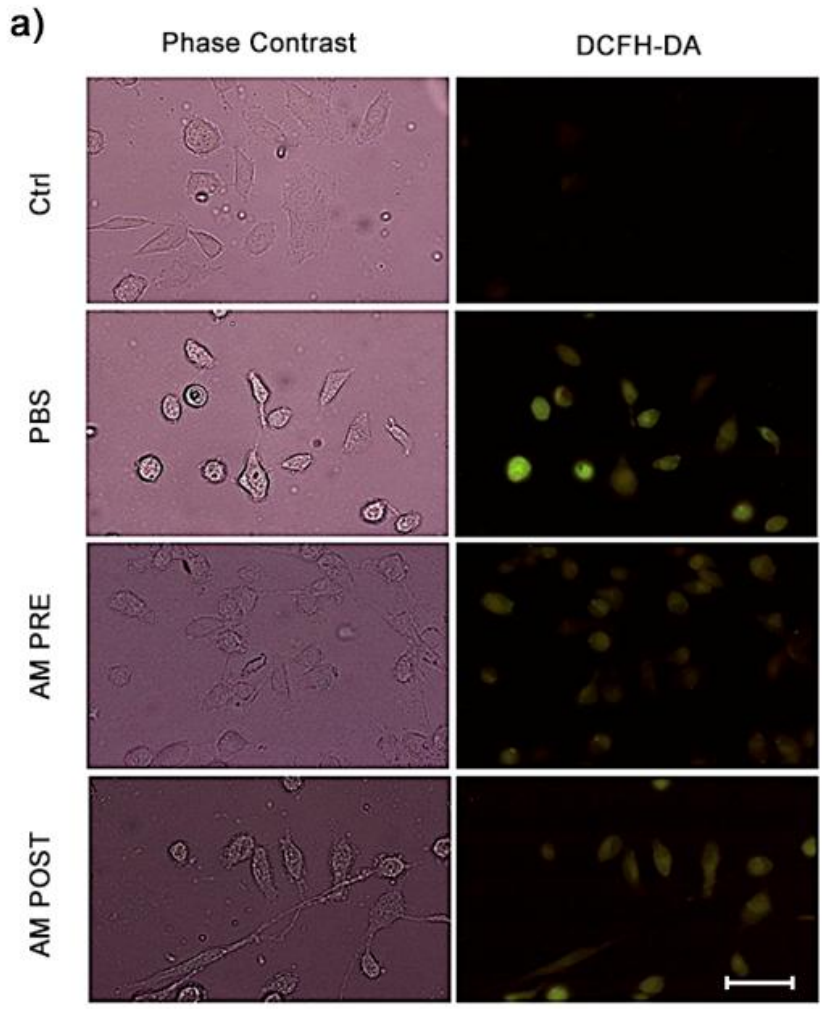
Fig. 2

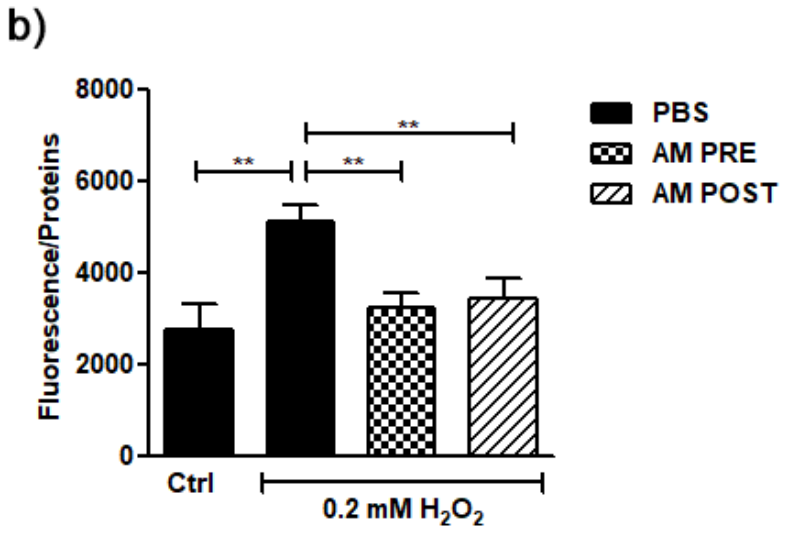


Viability of ARPE-19 cells after exposure to oxidative stress with amniotic membrane treatments. ARPE-19 cells were exposed to PBS (Ctrl), 0.2mM of H_2O_2 plus PBS (PBS), 0.2 mM of H_2O_2 with pre-treatment with AM (AM

PRE) or with post-treatment with AM (AM POST) and were evaluated using MTT (concentration of H_2O_2: 0.1, 0.2 and 0.3 mM). Data represent the mean value ± SEM. Bars indicate each significant comparison (*) indicate significant differences (***p < 0.001; **p < 0.01; *p < 0.05).

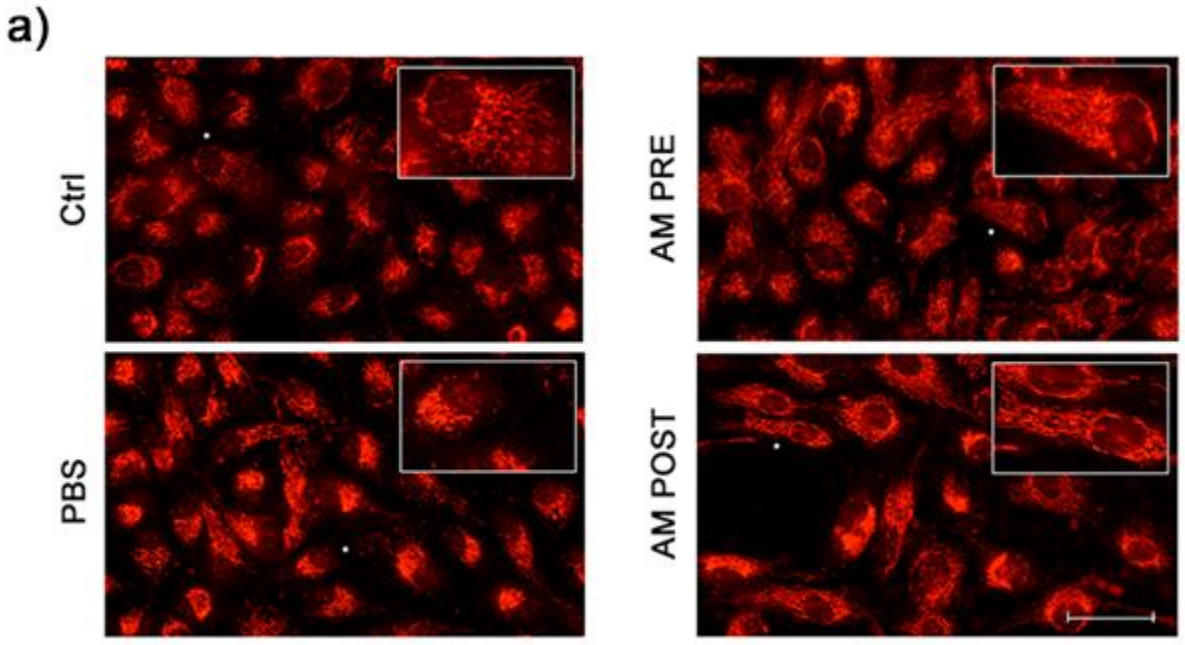
Fig. 3



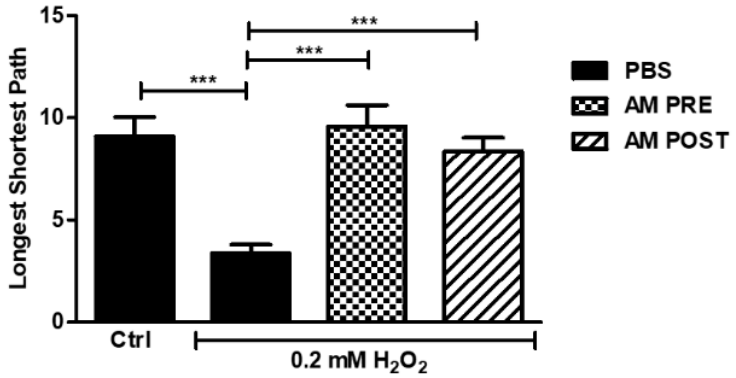


Effect of AM on ROS generation in H_2O_2-exposed ARPE-19 cells. a) Representative microphotographs of cells labeled with DCFH-DA after exposure to PBS (Ctrl), 0.2mM of H_2O_2 plus PBS (PBS), 0.2 mM of H_2O_2 with pre-treatment with AM (AM PRE) or with post-treatment with AM (AM POST). Scale bar: 50 µm. b) Quantitative analysis of ROS levels showing the same conditions. Data are expressed as fluorescence intensity normalized to the protein concentration. Bars indicate each significant comparison and asterisks indicate significant differences between the indicated groups (**$p < 0.01$).

Fig. 4

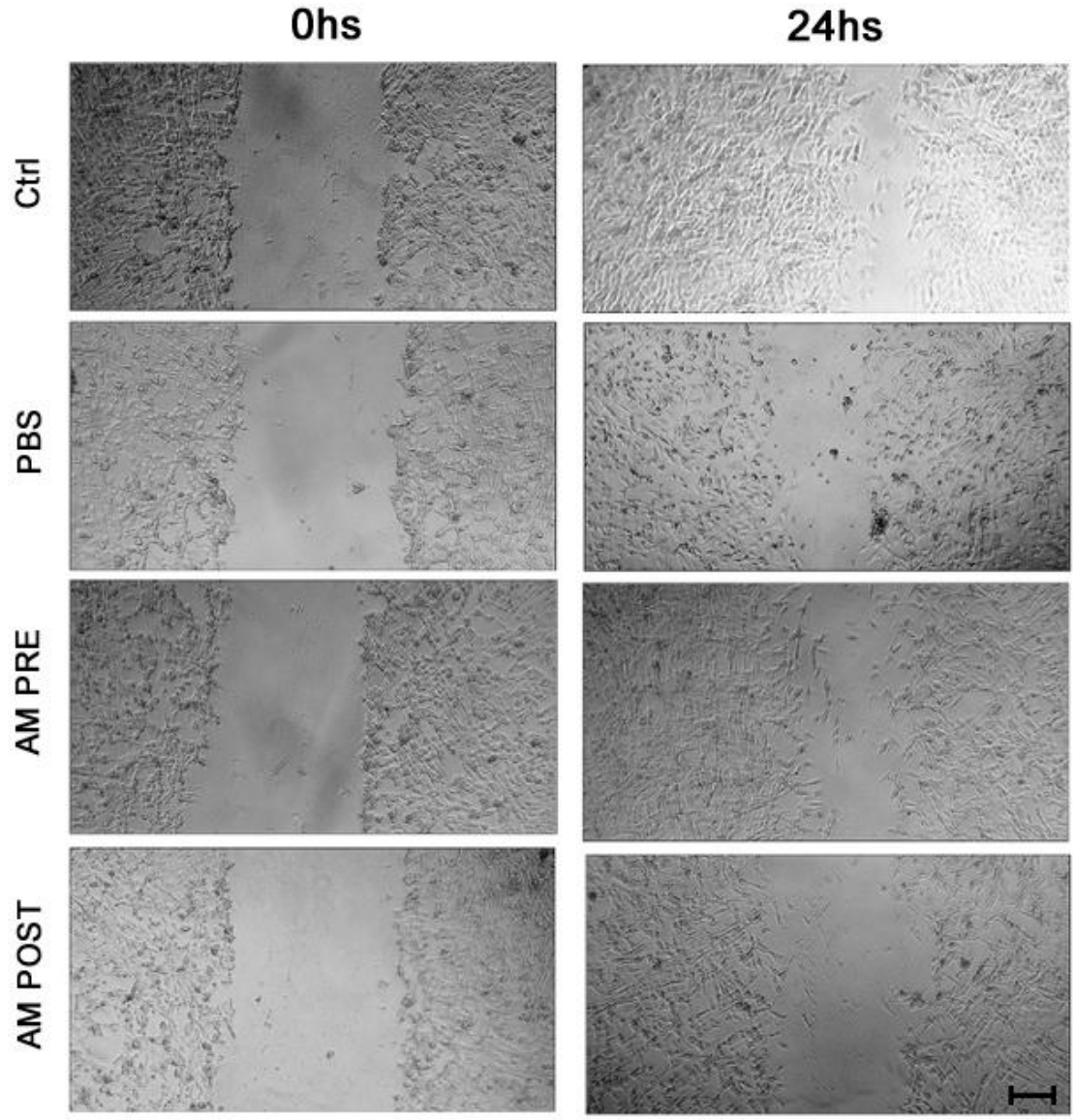


b)



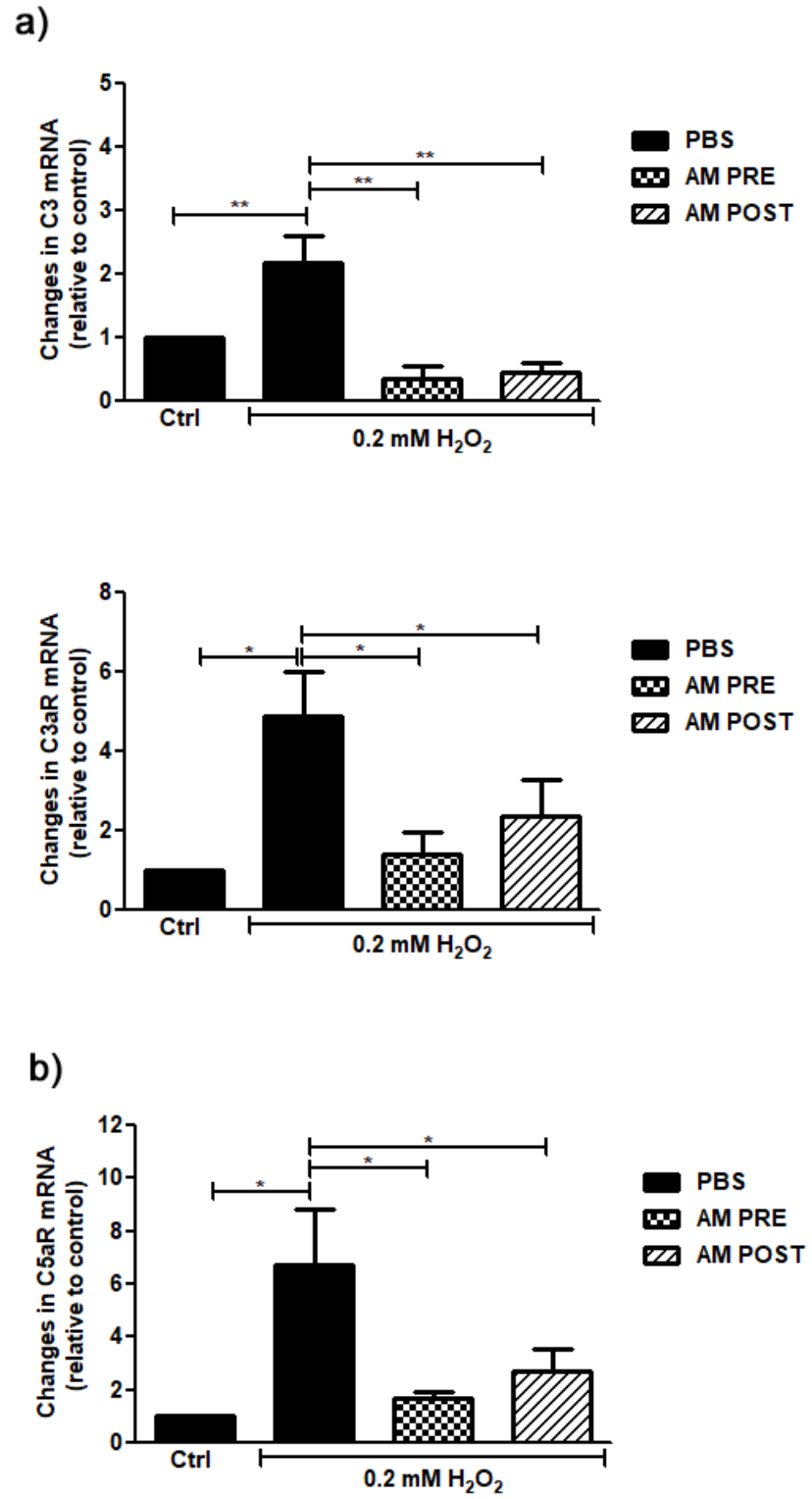
Mitochondrial morphology in H_2O_2-exposed ARPE-19 cells with AM treatments. a) MitoTracker Red fluorescence images show mitochondria of PBS (Ctrl), 0.2mM of H_2O_2 plus PBS (PBS), 0.2 mM of H_2O_2 with pre-treatment with AM (AM PRE) or with post-treatment with AM (AM POST). Scale bar: 50 μm. Asterisks indicate the inset area with 2x digital magnifications. b) Quantitative analysis of mitochondrial fragmentation using the Longest Shortest Path (LSP). A significant reduction in LSP values in PBS with respect to Ctrl indicates a shift toward a fragmented morphology, while AM treatments (AM PRE and AM POST) significantly preserved or restored the path length as control situation. Bars indicate each significant comparison and asterisks indicate significant differences between the indicated groups (*** $p < 0.001$).

Fig. 5



Monolayer integrity in H_2O_2-exposed ARPE-19 cells with AM treatments. The ARPE-19 cells were treated with PBS (Ctrl), 0.2mM of H_2O_2 plus PBS (PBS), 0.2 mM of H_2O_2 with pre-treatment with AM (AM PRE) or with post-treatment with AM (AM POST) using a wound-healing assay. Phase-contrast microphotographs were captured immediately after wounding (0 h) and 24 h post-treatments (24 h). Scale bar: 100 μm.

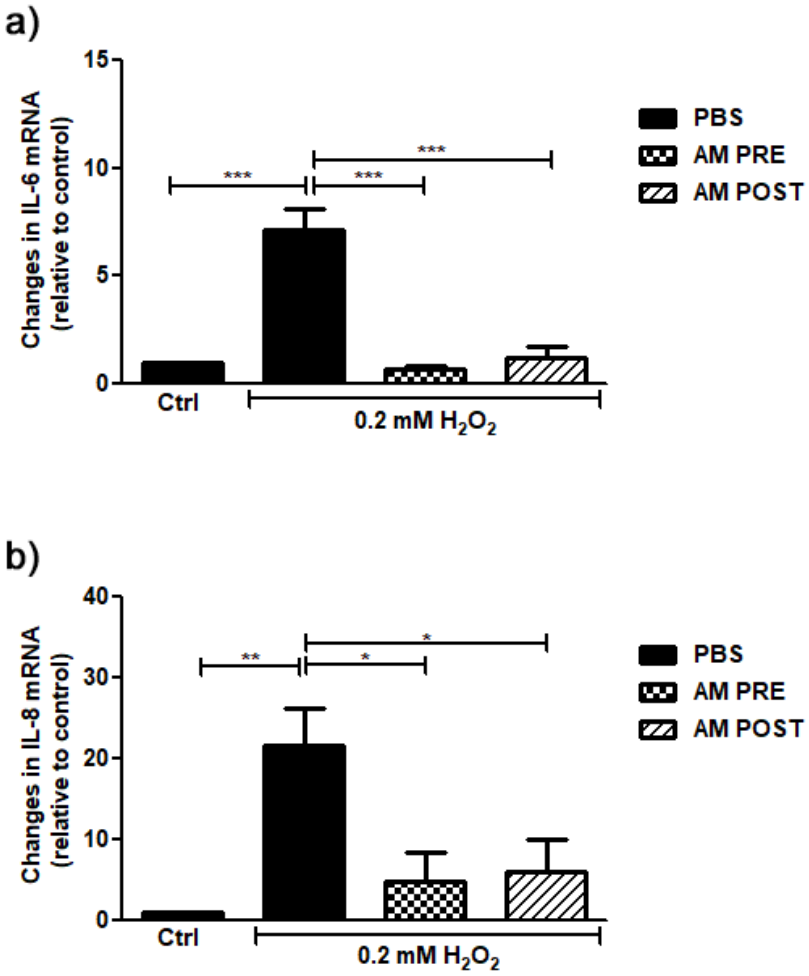
Fig. 6



Gene expression of complement components in H_2O_2-exposed ARPE-19 cells. Gene expression of C3, C3aR (a) and C5aR (b) on ARPE-19 cells exposed to PBS (Ctrl), 0.2mM of H_2O_2 plus PBS (PBS), 0.2 mM of H_2O_2 with

pre-treatment with AM (AM PRE) or with post-treatment with AM (AM POST) were analyzed using RT-qPCR with ΔΔCT method compared with Control (Ctrl). The expression of GAPDH gene was used as endogenous control. Data are expressed as mean values + SEM, n = 3. Bars indicate each significant comparison and asterisks (*) indicate significant differences with respect to control condition (**p < 0.01; *p < 0.05).

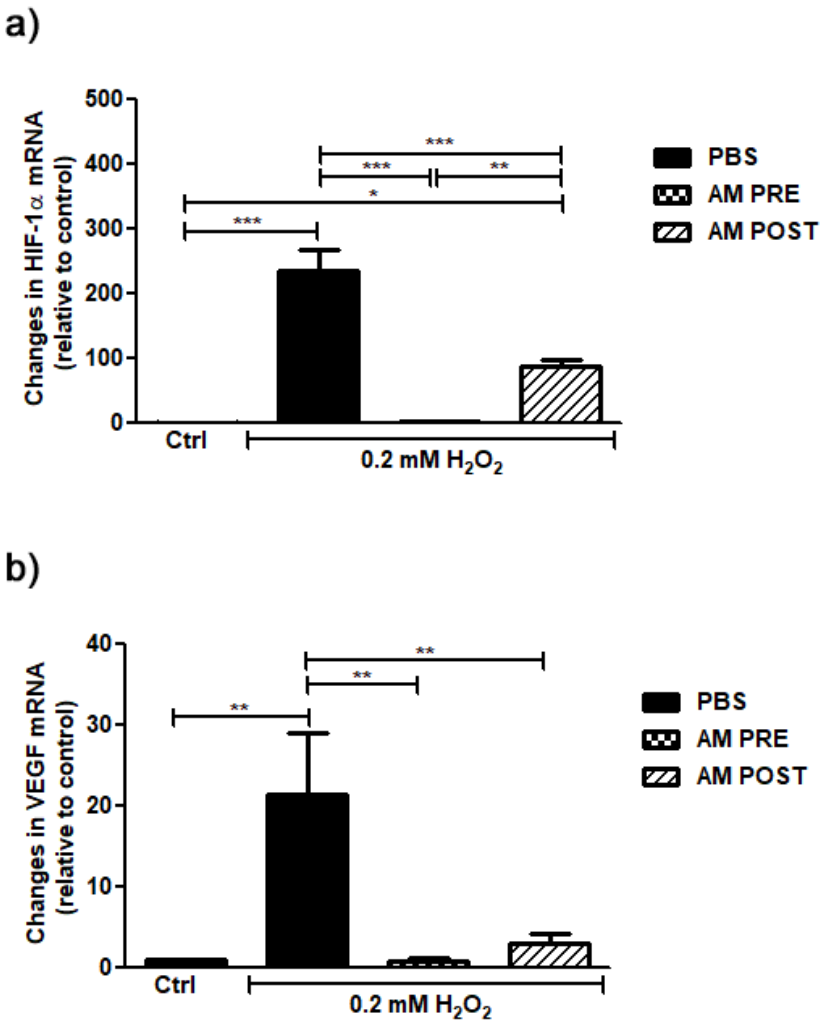
Fig. 7



Gene expression levels of pro-inflammatory interleukins in H_2O_2-exposed ARPE-19 cells. Gene expression of IL-6 (a) and IL-8 (b) on ARPE-19 cells exposed to PBS (control, Ctrl), 0.2mM of H_2O_2 plus PBS (PBS), 0.2 mM of H_2O_2 with pre-treatment with AM (AM PRE) or with post-treatment with AM (AM POST) were analyzed using RT-qPCR with ΔΔCT method. Control were used as reference sample and GAPDH gene expression was used as endogenous control. Data are expressed as mean values + SEM, n = 3. Bars indicate each significant comparison

and asterisks (*) indicate significant differences with respect to control condition (***p < 0.001; **p < 0.01; *p < 0.05).

Fig. 8



Expression of pro-angiogenic factors in H_2O_2-exposed ARPE-19 cells. Gene expression of HIF-1α (a) and VEGF (b) in ARPE-19 cells exposed to PBS (control group, Ctrl), 0.2mM of H_2O_2 plus PBS (PBS), 0.2mM H_2O_2 with pre-treatment of AM (AM PRE group) or with post-treatment of AM (AM POST group) were analyzed using RT-qPCR with ΔΔCT method. Control values were used as reference sample and GAPDH gene expression was used as endogenous control. Data are expressed as mean values + SEM, n = 3. Bars indicate each significant comparison and asterisks (*) indicate statistically significant differences with respect to control condition (***p < 0.001; **p < 0.01; *p < 0.05).