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MODULATION OF INFLAMMATION AND ROS IN ARPE-19 CELLS TREATED WITH hAM DERIVATIVES UNDER OXIDATIVE STRESS CONDITIONS

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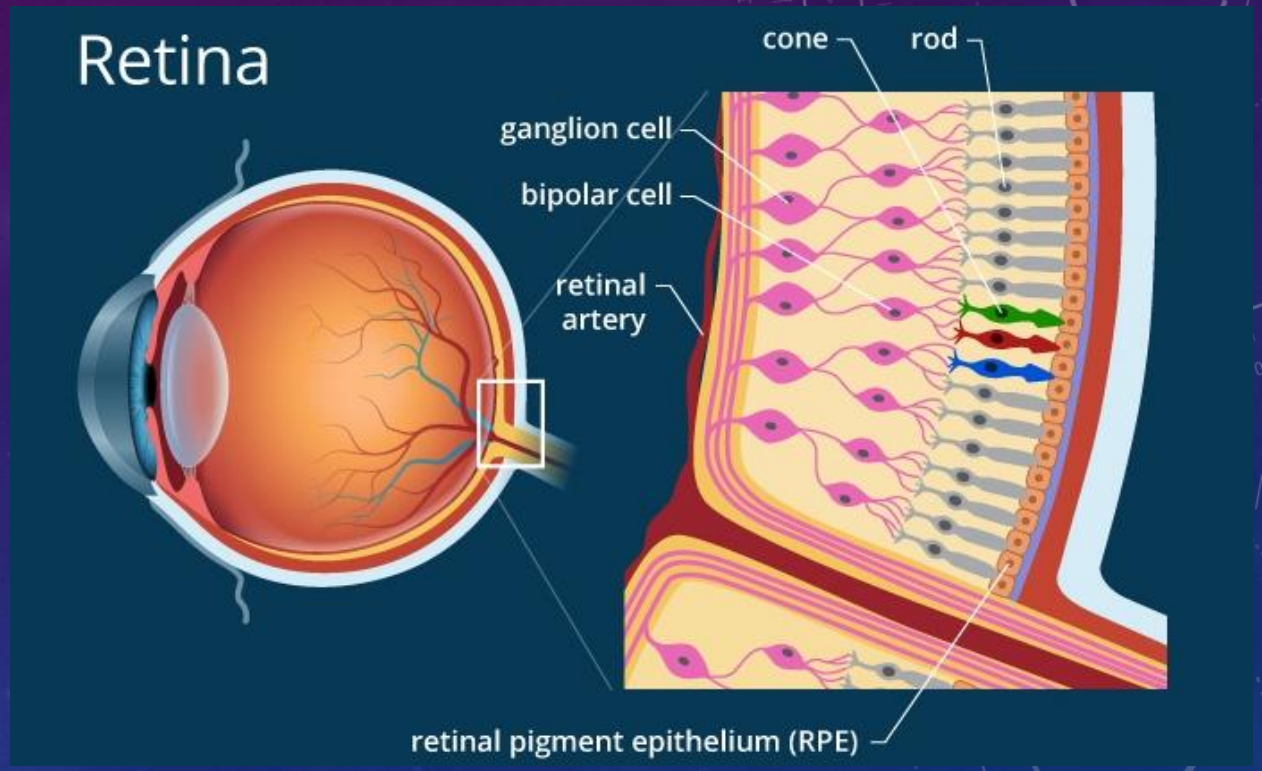
3-CONICET

RETINAL PIGMENT EPITHELIUM AND MDA

- The retinal pigmented epithelium (RPE) plays a crucial structural and supportive role in maintaining photoreceptor homeostasis and functionality.
- RPE cells are one of the primary cells affected in retinal injuries such as Age-related macular degeneration (AMD).
- AMD significantly impacts older adults, severely diminishing their quality of life.
- While treatments are available for the wet variant of AMD, the dry variant currently lacks such options.
- Increasing the intake of antioxidant vitamins and minerals through diet or supplements is among the few interventions shown to slow the progression to more advanced stages of dry AMD.

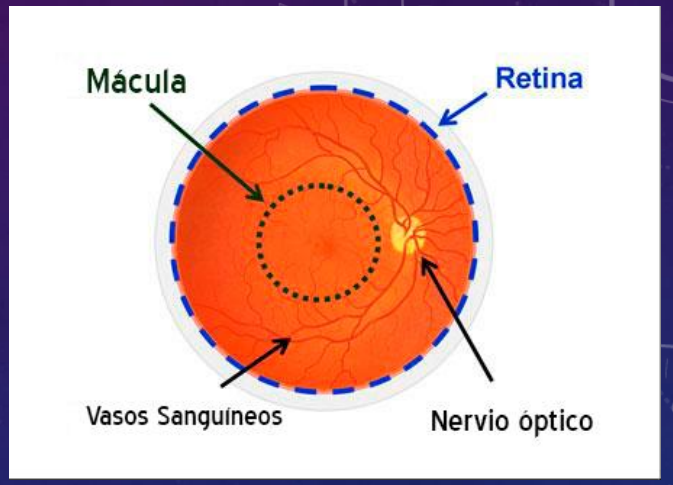


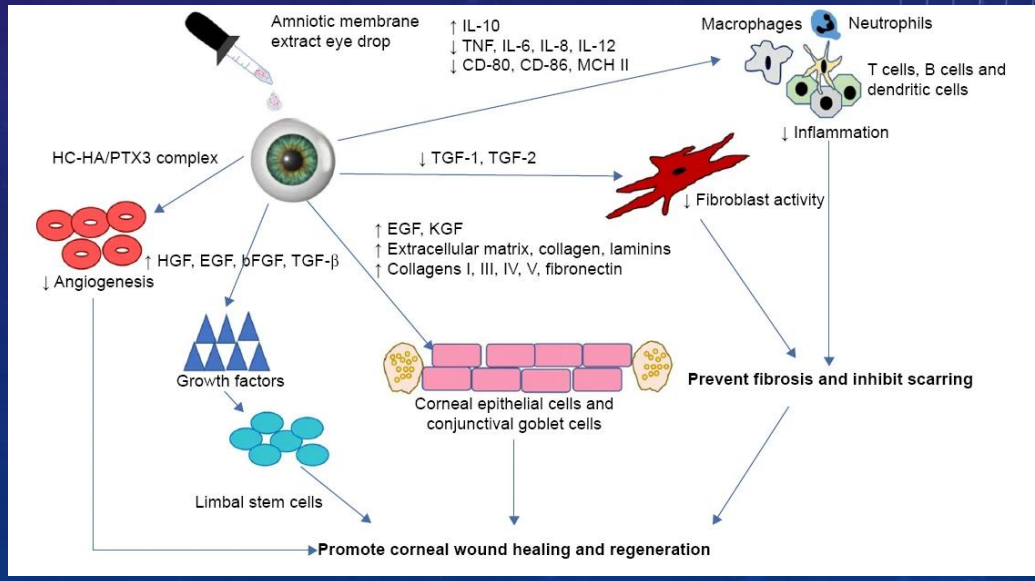
https://ocularis.es

It´s imperative to discover effective therapies for dry variant of AMD

HUMAN AMNIOTIC MEMBRANE (hAM)

- Research has identified that at least five pathways are affected in age-related macular degeneration (AMD). These include:
  - Inflammatory pathways (complement cascade)
  - Extracellular matrix remodeling pathways
  - Lipid metabolism
  - Angiogenesis
  - Response to oxidative stress
- The human amniotic membrane is an avascular tissue that forms the innermost layer of the fetal membrane.
- Its molecular composition is abundant in bioactive molecules, exhibiting proven antioxidant, anti-inflammatory, and anti-degenerative properties.
- Although it has been extensively utilized for the repair of ocular surface lesions, its protective effects on retinal layers are still relatively underexplored.





OBJECTIVES

This study explores the effects of hAM on retinal pigment epithelium (RPE) cells under oxidative stress conditions. Our focus is on:

- Survival and migratory capacity
- Oxidative levels
- Inflammatory responses

MATERIALS AND METHODS

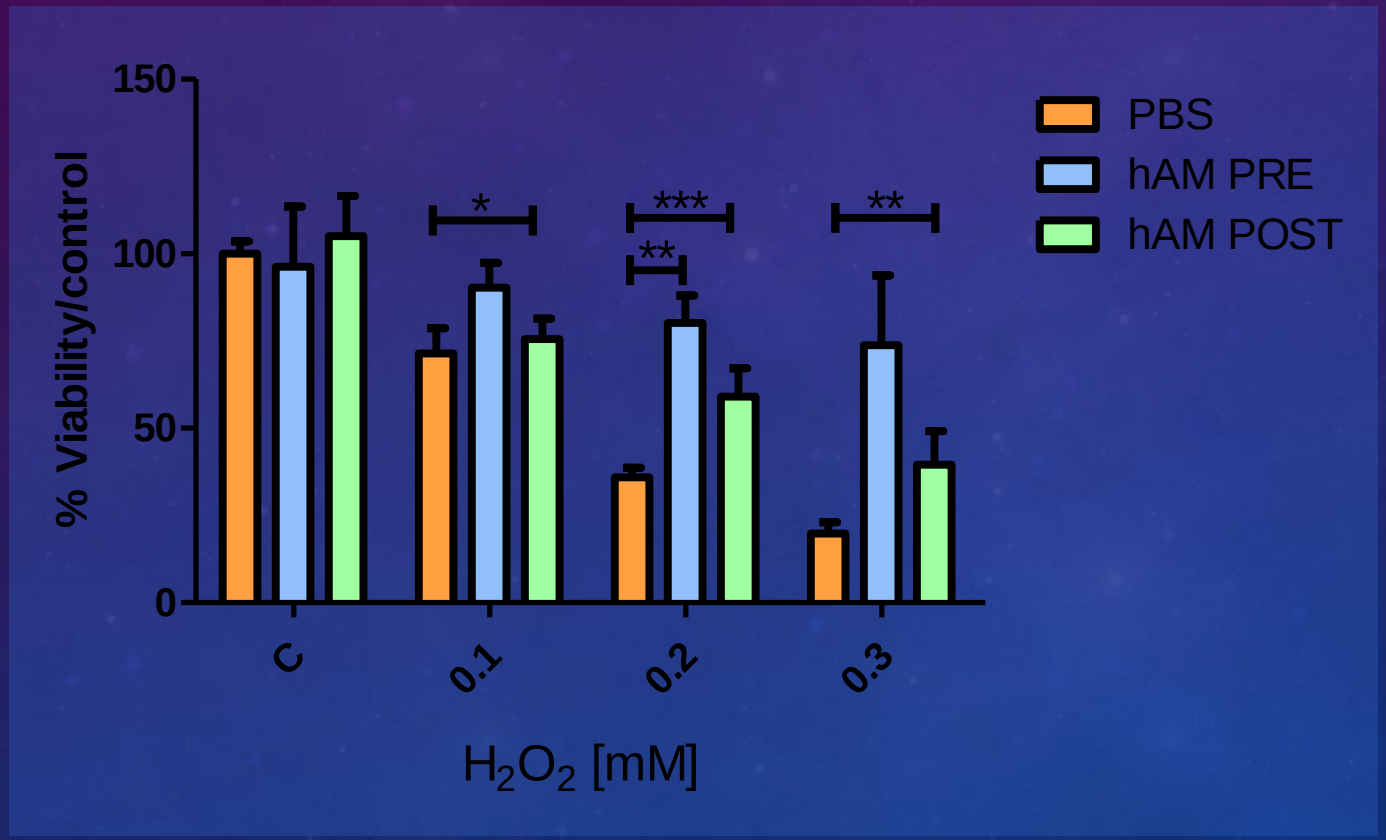
Human Amniotic Membrane

- Full term human placentas and fetal membranes were collected shortly after delivery. The amnion was separated from the rest of chorion and washed repeatedly with sterile saline solution with antibiotics.
- hAM fragments were placed in DMEM at a 1:1 volume-to-weight ratio and homogenized using a mechanical mixer. The homogenate was then centrifuged at 10,000 rpm for 10 minutes. The supernatant was filtered through a 0.2 µm pore filter and frozen for later use. Total protein content was assessed using the Bradford method.
- *In vitro*, ARPE-19 cells were exposed to hydrogen peroxide (H_2O_2) to induce oxidative stress conditions.
- We examined the effects of pre-treatment and post-treatment with hAM using MTT and propidium iodide (PI) assays to explore cell survival and death levels and wound healing assays to explore the migratory activities of the cells. Reactive oxygen species (ROS) were measured with 2,7-dichlorofluorescin diacetate (DCF-DA).
- In THP-1 cells subjected to LPS, IL-6 and IL-10 were measured with ELISA following treatment with hAM derivatives.

hAM TREATMENT ON CELL VIABILITY OF ARPE-19 UNDER OXIDATIVE STRESS CONDITIONS

- Cell viability was analyzed using the MTT assay.
- ARPE-19 cells were plated in 24-well culture dishes at a density of 3×10^4 cells per well. After 24 hours, the cells were treated with H_2O_2 (0.1-0.3 mM) in DMEM-F12 supplemented with 1% FBS.
- For hAM treatment, one group of wells received hAM 3 hours after the addition of H_2O_2, while another group received hAM prior to the addition of H_2O_2. The plates were read using a multi-plate reader set to 570 nm.
- We added 0,2ug/ul of hAM per well.

hAM TREATMENT ON CELL VIABILITY OF ARPE-19 UNDER OXIDATIVE STRESS CONDITIONS



- As previously established, ARPE-19 cells showed a decrease in viability that is proportional to the concentration of H_2O_2.
- Pre-treatment with hAM almost completely abolished the effects of H_2O_2.
- Post-treatment with hAM significantly reduced the impact of H_2O_2 at intermediate concentrations of this agent.

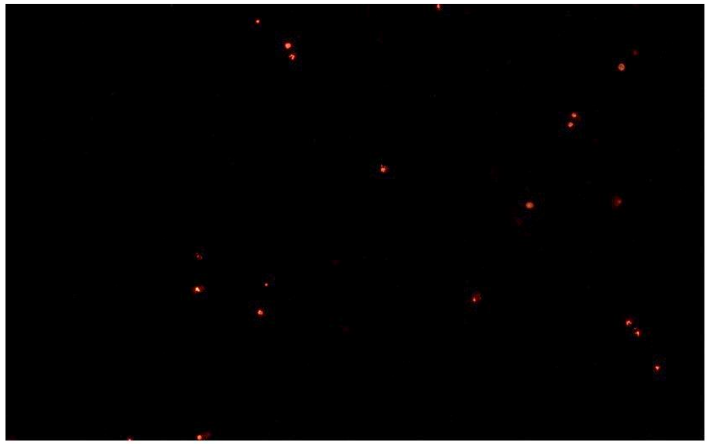
hAM TREATMENT ON CELL DEATH OF ARPE-19 UNDER OXIDATIVE STRESS CONDITIONS

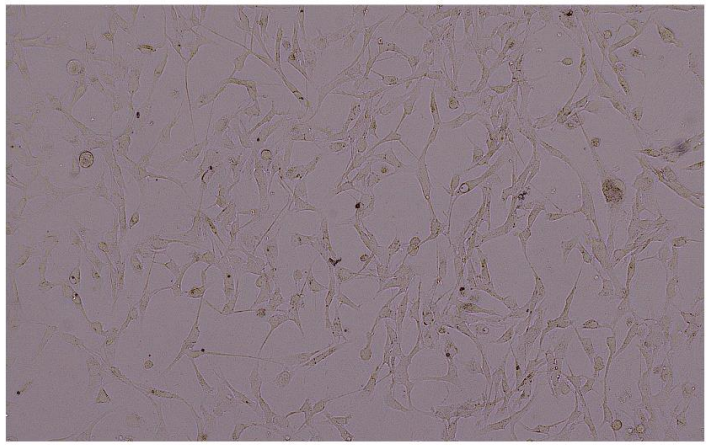
- ARPE-19 cells were seeded onto 12 mm coverslips and subjected to treatments similar to those in previous assays. Cell death was analyzed with PI staining. PI passes through permeable cell membranes of necrotic cells and stains double-stranded DNA.
- After the experimental treatment, the cell culture medium was removed and replaced with medium containing 50 µg of IP. The cells were then washed and the coverslips were mounted for examination using a fluorescent microscope equipped with a camera and counted.

IP

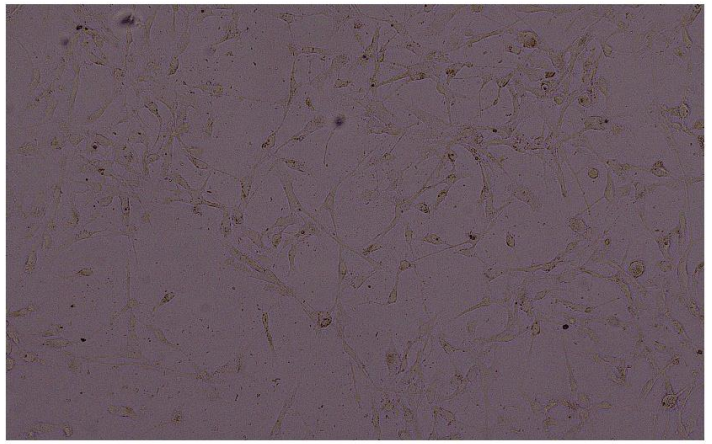
Phase Contrast

0,2mM H_2O_2

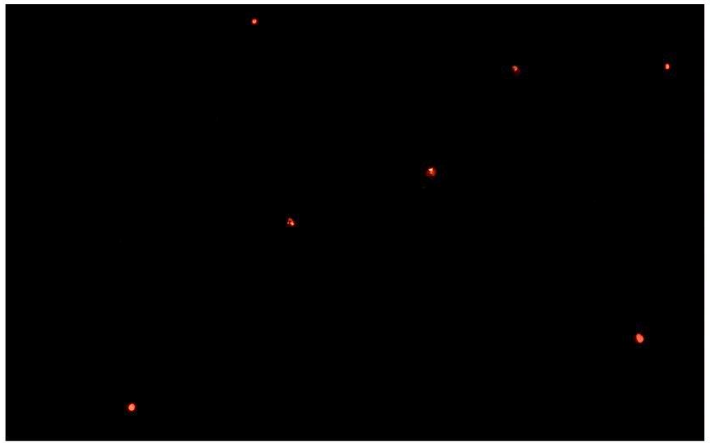


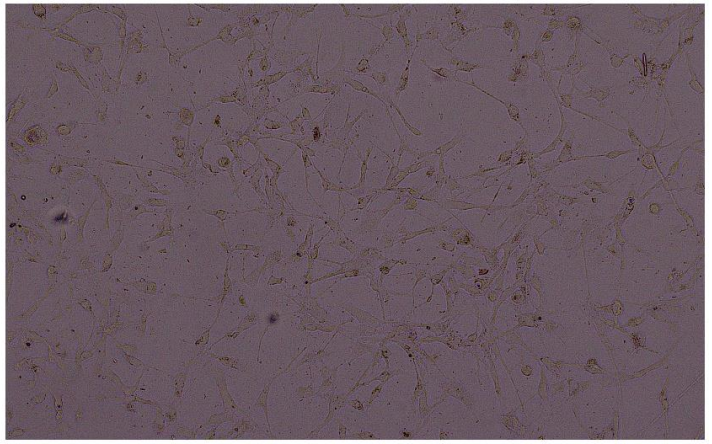


hAM previous 0,2mM H_2O_2



hAM post 0,2mM H_2O_2

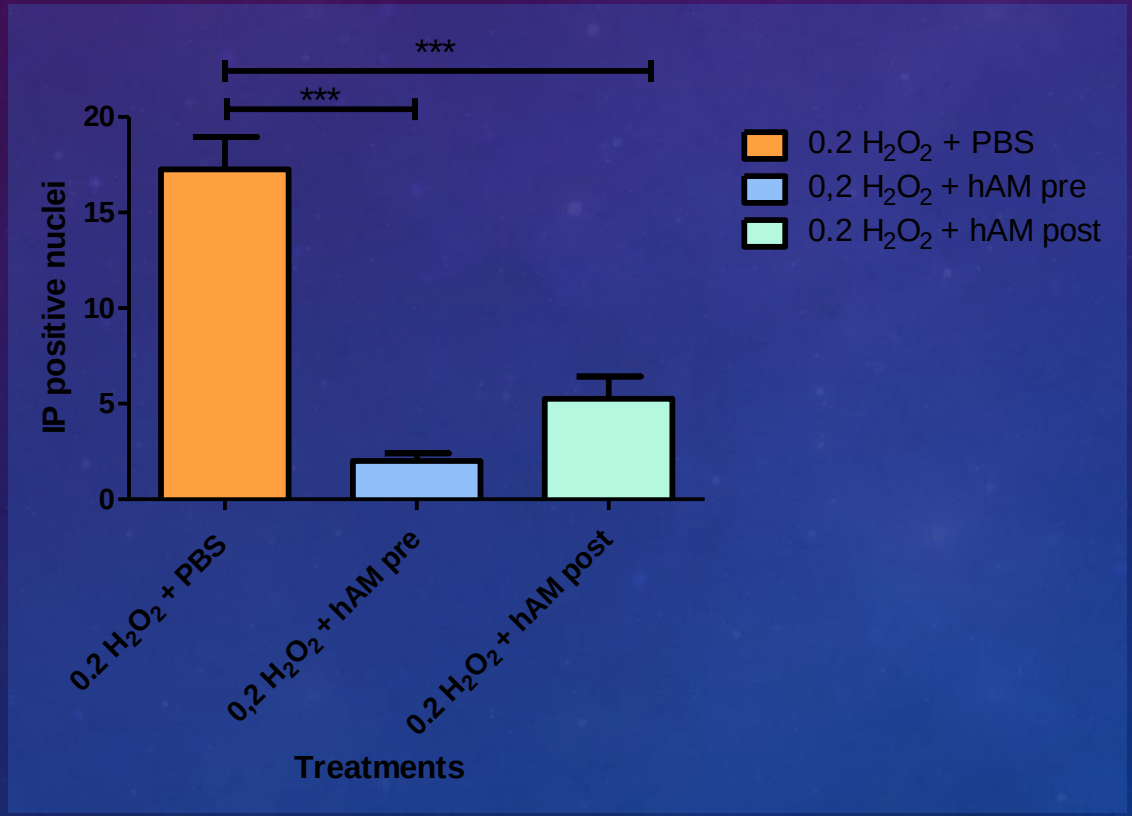




Exposure to H_2O_2 produced that IP stain could penetrated in ARPE-19 death cells.

Both pre- and post-treatment with hAM reduced the amount of ARPE-19 cell nuclei stained with IP.

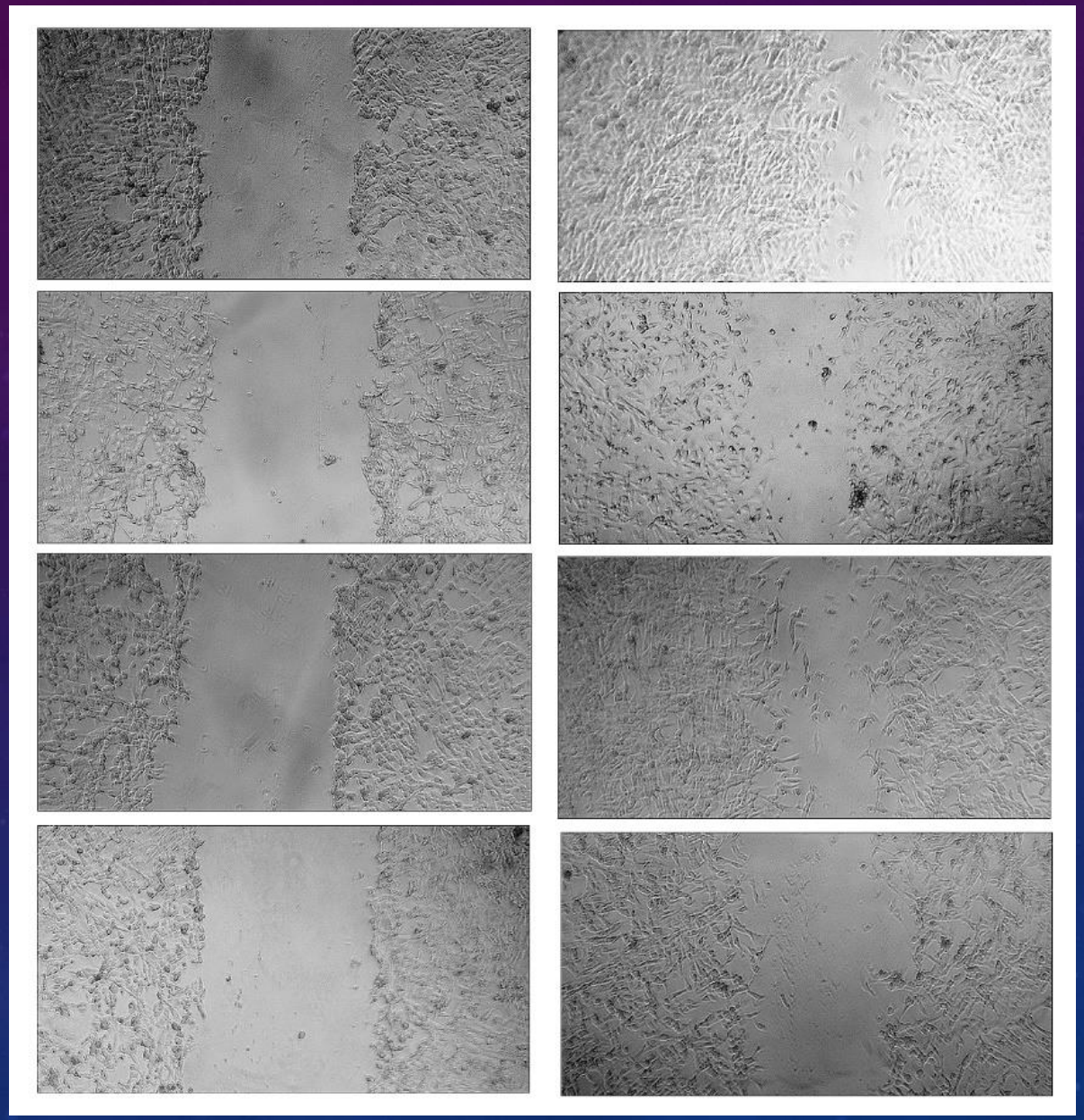
hAM TREATMENT ON CELL DEATH OF ARPE-19 UNDER OXIDATIVE STRESS CONDITIONS



The number of IP positive nuclei in 10 fields from two different experiments shows a significant reduction in cell death with previous and subsequent treatment with hAM compared to H_2O_2 with PBS.

hAM TREATMENT ON WOUND ASSAY OF ARPE-19 CELLS UNDER OXIDATIVE STRESS CONDITIONS

- ARPE-19 cells were seeded in a 24-well dish until a confluent monolayer was achieved. A straight line was drawn using a P200 pipette tip. Similar treatments to the viability assay were performed, using only 0.2 mM of H_2O_2.
- Microphotographs were taken at 24 and 48hs after the scratch.
- The size of the scratch area was analyzed.



The ARPE-19 cells in the scar begin to detach and degenerate under H_2O_2 treatment.

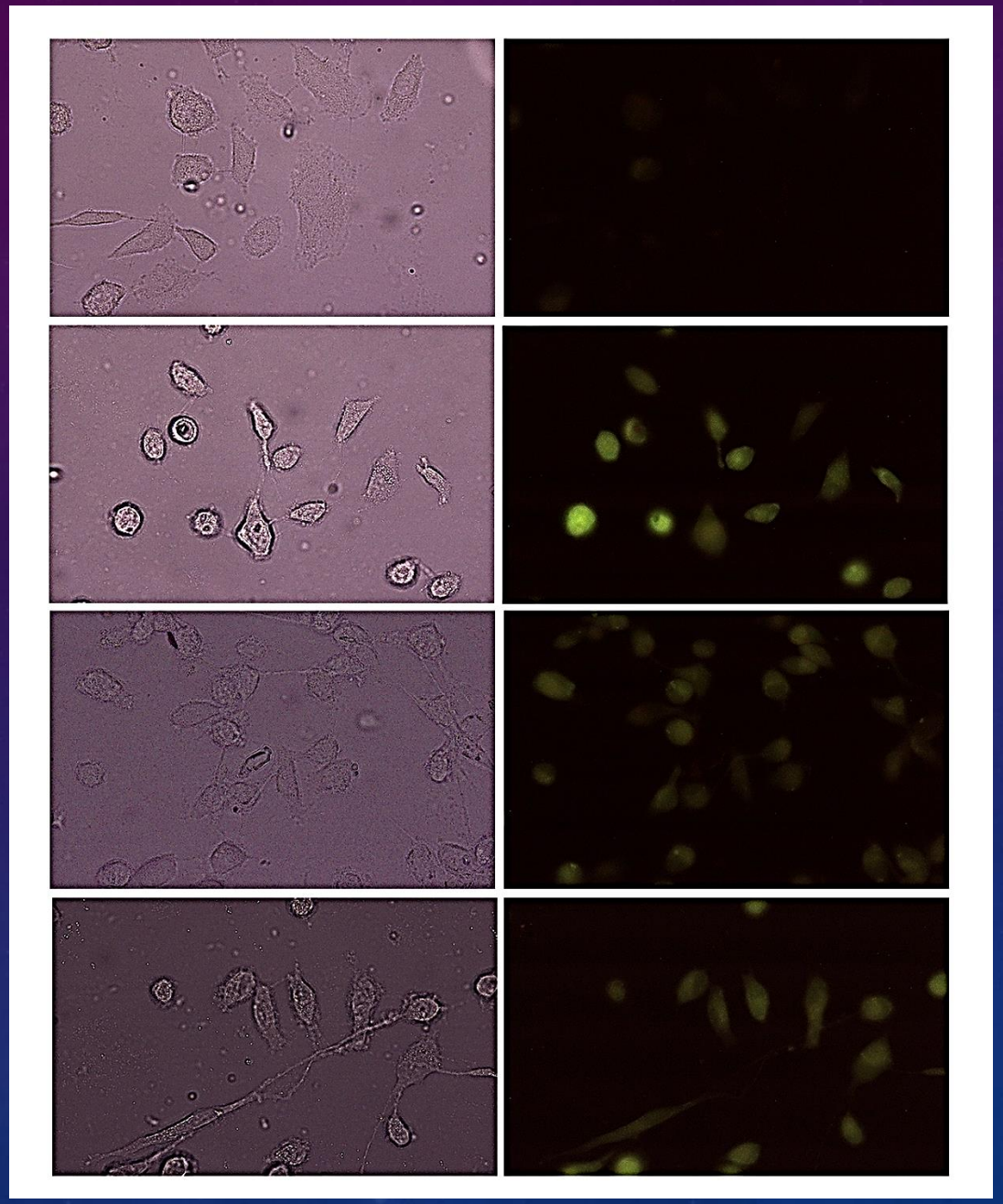
However, the addition of hAM prior to H_2O_2 effectively blocked the effects of this agent, prompting the cells to migrate to the scar area.

In contrast, adding hAM after H_2O_2 treatment also inhibited its effects, but the cells did not migrate to the scar area.

hAM TREATMENT ON REACTIVE OXYGEN SPECIES (ROS) PRODUCTION BY ARPE-19 UNDER OXIDATIVE STRESS CONDITIONS

- ROS production was measured using the probe 2',7'-Dichlorofluorescin diacetate (DCFH-DA), which can cross the cell membrane and, upon oxidation, is converted into a fluorescent compound.
- After the experimental treatment, the cell culture medium was removed and replaced with medium containing 10 µM DCFH-DA, followed by incubation for 30 minutes at 37 °C.
- The cells were then washed and the coverslips were mounted for examination using a fluorescent microscope equipped with a camera.
- In a separate group of wells, after incubation with DCFH-DA, the cells were harvested using trypsin, and their fluorescence was measured with a fluorimeter.

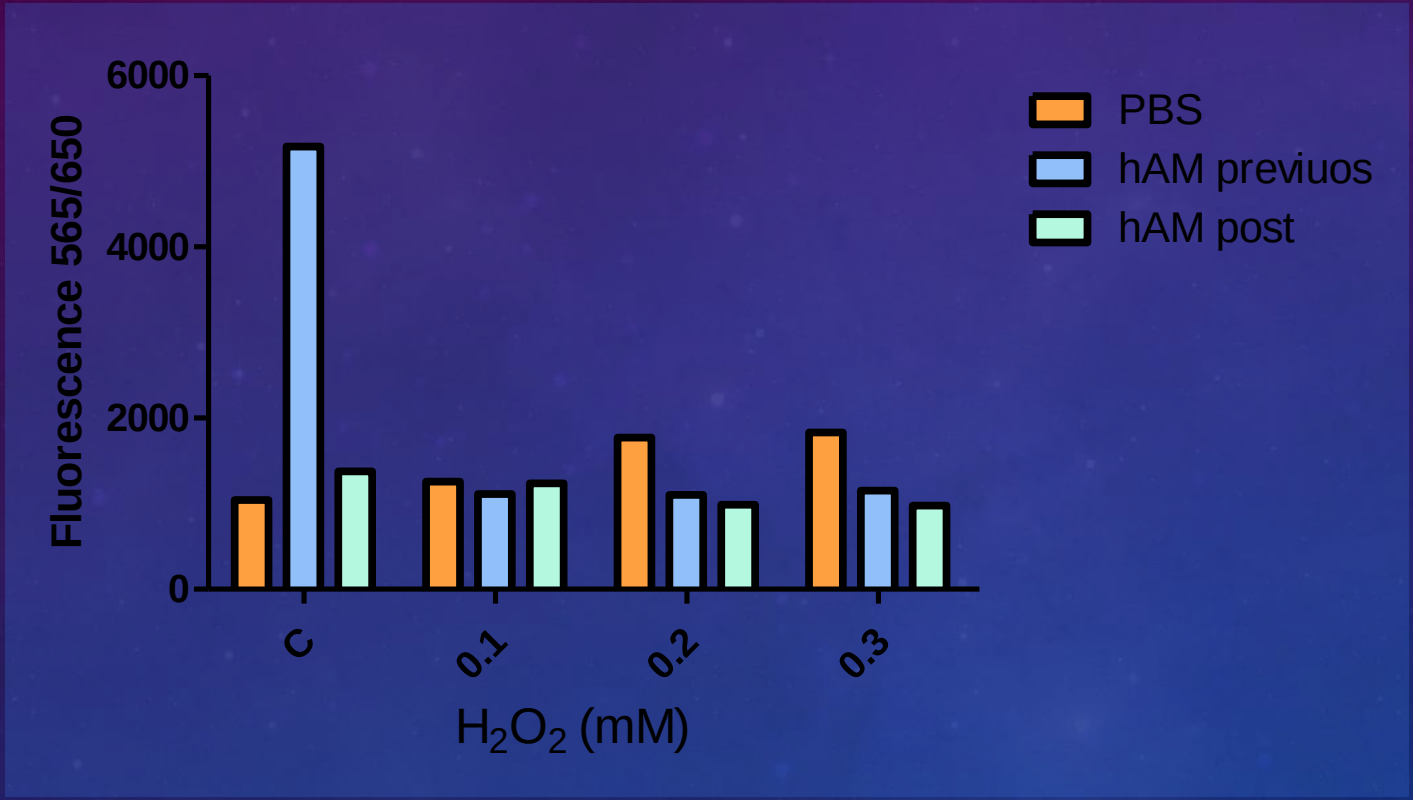
ROS generation in RPE cells exposure to H_2O_2 and pre or post treated with hAM



Exposure to H_2O_2 induced increases in fluorescence levels compared to the control situation.

Both pre- and post-treatment with hAM resulted in a reduction of fluorescence levels.

ROS GENERATION IN RPE CELLS EXPOSED TO H_2O_2 AND TREATED PRE- OR POST- WITH HAM.



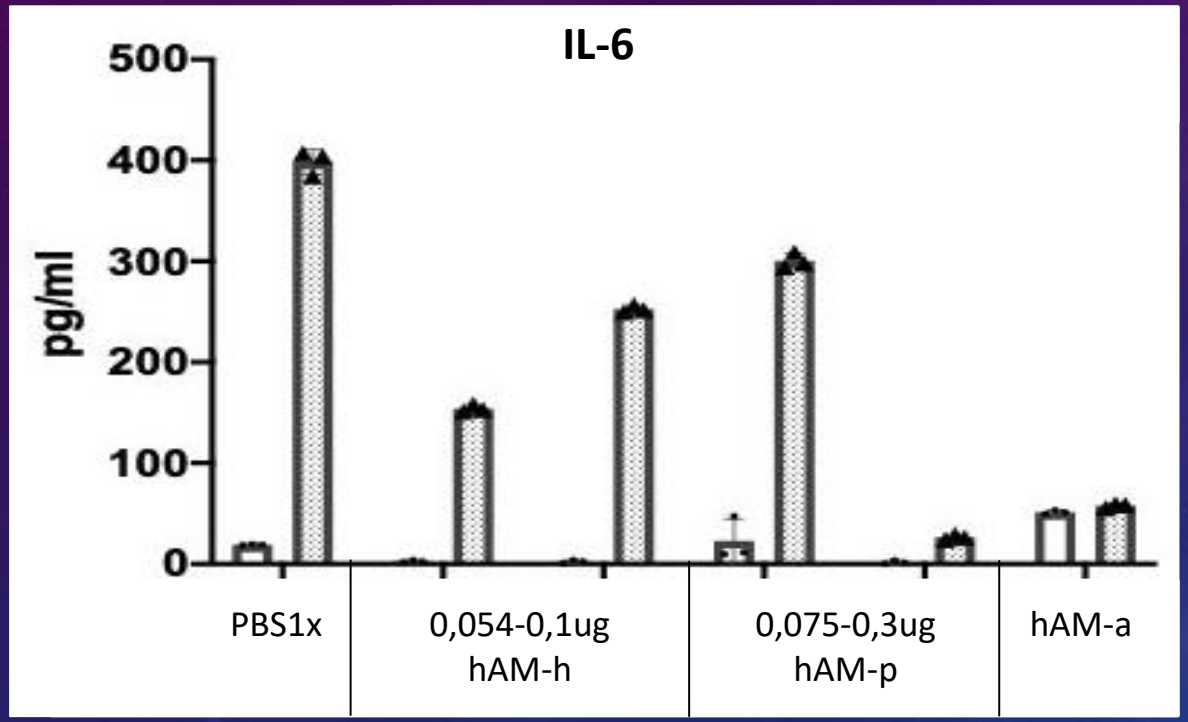
Exposure to H_2O_2 induced increases in fluorescence levels compared to the control situation.

Both pre- and post-treatment with hAM resulted in a reduction of fluorescence levels.

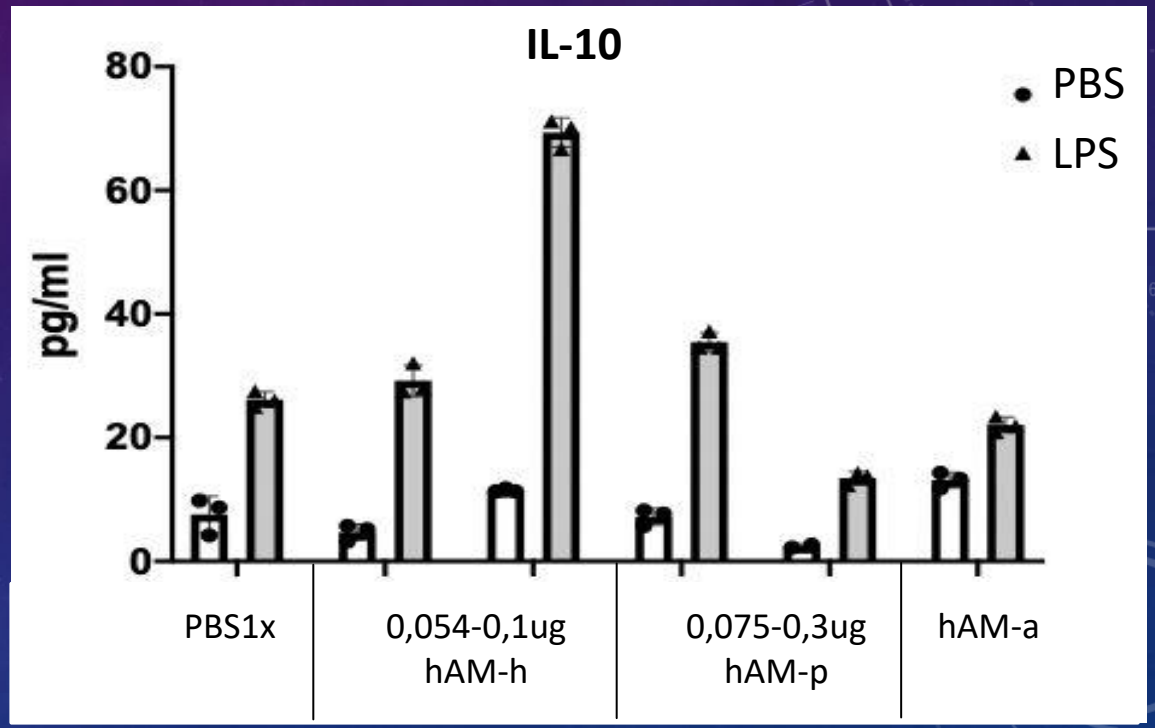
hAM treatment on anti-inflammatory and inflammatory signals in THP-1 cells under LPS treatment

- THP-1 monocytic cells were activated with PMA for 72 hours and then exposed to LPS along with hAM derivatives. After 20 hours, the levels of IL-6 and IL-10 were determined using ELISA kits.

hAM DERIVATIVES REDUCED PRO-INFLAMMATORY IL-6 AND INCREASED ANTI-INFLAMMATORY IL-10 IN THP-1 CELLS TREATED WITH LPS



The secretion of pro-inflammatory IL-6 induced by LPS is negatively modulated by all the tested hAM derivatives.



The secretion of anti-inflammatory IL-10 increases upon the addition of hAM derivatives.

CONCLUSIONS

Our research indicates that hAM derivatives protect RPE cells from oxidative stress, functioning not only as a preventive agent but also as a therapeutic intervention once metabolic imbalance has already occurred.

Its effects may be directly related to improvements in the dysfunction of certain pathways affected in conditions such as AMD. For instance, hAM derivatives induced a reduction in cell death, a decrease in ROS levels, an increase in anti-inflammatory and a reduction in pro-inflammatory signals.