Original Article

Effects of hydroquinone on retinal and vascular cells *in vitro*

Ashish Sharma[1,2], Jayaprakash A Patil[1,3], Ana L Gramajo[4], Gail M Seigel[5], Baruch D Kuppermann[1], Cristina M Kenney[1]

Aim: To explore the molecular pathophysiology that might explain the epidemiologic association between cigarette smoke and age-related macular degeneration (AMD) by examining the effects of hydroquinone (HQ), a toxic compound present in high concentration in cigarette smoke-related tar, on human retinal pigment epithelial cells (ARPE-19), rat retinal neurosensory cells (R-28), and human microvascular endothelial cells (HMVEC). **Materials and Methods:** ARPE-19, R-28, and HMVEC were treated for 24 h with four different concentrations of HQ (500 μM, 200 μM, 100 μM, 50 μM). Cell viability, caspase-3/7 activation, DNA laddering patterns, and lactate dehydrogenase (LDH) levels were analyzed. **Results:** At 50 μM HQ, R-28 cells showed a significant decrease in cell viability compared with the dimethyl sulfoxide (DMSO)-treated controls. At the 100–500 μM concentrations, all three cell lines showed significant cell death ($P < 0.001$). In the ARPE-19, R-28, and HMVEC cultures, the caspase-3/7 activities were not increased at any of the HQ concentration. **Conclusion:** Our findings suggest that the mechanism of cell death in all three cell lines was through non-apoptotic pathway. In addition, neuroretinal R-28 cells were more sensitive to HQ than the ARPE-19 and HMVEC cultures.

Key words: Apoptosis, caspase-3/7, cigarette smoke toxicant, hydroquinone

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Age-related macular degeneration (AMD) is the most common cause of registered visual handicap in the older age group in North America.[1] AMD is a degenerative disorder of the retinal pigment epithelium (RPE) and neurosensory retina, but its exact etiology is unknown. Cigarette smoke contains an abundance of potential oxidants including many of those belonging to the quinone family, which are present in the tar of cigarette smoke. Hydroquinone (HQ) is the most abundant quinine present in cigarette tar.[2,3] HQ is found in varying amounts from 100 to 300 μg per cigarette in non-filter cigarettes.[4] Each smoked cigarette can deposit as much as 100 μg of HQ into the lungs.[5] High levels of HQ can be detected in the plasma and urine of smokers, indicating that it can enter the circulation via the lungs and can interact with cellular targets throughout the body.[3] Many studies reported hepatotoxic and nephrotoxic properties of HQ.[6,7]

A number of clinical and epidemiological studies have shown a strong correlation between smoking and AMD.[8-10] In this study, we examined the effects of HQ at the cellular level. Although the pathogenesis of AMD includes different clinical signs, the degeneration of RPE cells is often observed at early stages of the disease with subsequent changes in neurosensory cells and vascular cells.[11] Therefore, we used human retinal pigment epithelial cells (ARPE-19), rat retinal neurosensory cells (R-28), and human microvascular endothelial cells (HMVEC) in this study.

In disease processes, many parallel pathways ultimately lead to cell death. Apoptosis refers to programmed cell death mediated by a family of cysteine proteases known as caspases. Among all caspases, activation of caspase-3/7 is a downstream event that leads to cell death. Non-apoptotic cell death can occur in many forms such as autophagy, aponecrosis or necroptosis and necrosis.[12] Our data suggest that non-apoptotic cell death may be an important mechanism by which smoking causes the onset and progression of AMD.

Materials and Methods

Cell culture

Cells from the human retinal pigment epithelial line ARPE-19 were obtained (ATCC, Manassas, VA, USA) for the study. Cells were grown in a 1:1 mixture (vol/vol) of Dulbecco's Modified Eagle's Medium with Ham's F-12 nutrient medium (DMEM F-12; Gibco, Carlsbad, CA, USA), nonessential amino acids 10 mM 1×, 0.37% sodium bicarbonate, 0.058% L-glutamine, 10% fetal bovine serum, and antibiotics (penicillin G 100 U/ml, streptomycin sulfate 0.1 mg/ml, gentamicin 10 μg/ml, amphotericin B (Fungizone) 2.5 μg/ml).

R-28 cells, which are rat embryonic precursor neurosensory retinal cells, were derived from postnatal day 6 rat retina in the laboratory of one of the authors (GMS). R-28 cells express genes characteristic of neurons,[13] as well as functional neuronal and glial properties.[14] R-28 cells were cultured in Dulbecco's modified Eagle's medium, high glucose (DMEM high glucose; Invitrogen-Gibco, Grand Island, NY, USA) with 10% fetal bovine serum, 1× minimum essential medium (MEM), 10 mM 1× nonessential amino acids, 0.37% sodium bicarbonate, and 10 μg/ml gentamicin.

[1]Department of Ophthalmology, Gavin S. Herbert Eye Institute, University of California, Irvine, CA, [2]Lotus Eye Care Hospital, Coimbatore, Tamilnadu, India, [3]Royal Lancaster Infirmary, University Hospitals of Morecambe NHS Trust, Lancaster, United Kingdom, [4]Departamento de Oftalmologia, Fundacion VER, Cordoba, Argentina, [5]University at Buffalo, Center for Hearing and Deafness, Buffalo, NY, USA

Correspondence to: Dr. Cristina M. Kenney, Department of Ophthalmology, University of California Irvine, Medical Center, 101 The City Drive, Orange, CA 92868. E-mail: mkenney@uci.edu

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HMVEC and their tissue culture reagents were obtained from Cascade Biologics, Inc. (Portland, OR, USA). The cells were acquired as proliferating quaternary cultures established from cryopreserved normal human microvascular endothelial cells isolated from adult human dermis. HMVEC were grown in Medium 131 supplemented with Microvascular Growth Supplement (MVGS) to support the plating and proliferation of cells. Medium 131 not supplemented with MVGS was used as serum-free medium for HMVEC. Before use, the tissue culture surfaces were coated with Attachment Factor (AF), a sterile 1× solution containing 0.1% gelatin.

The cells were plated onto 6-well and 24-well tissue culture plates (Becton Dickinson Lab ware, Franklin Lakes, NJ, USA) at 5×10^5 and 1.2 10^5 cells per well for cell viability and caspase-3/7 assay, respectively, and incubated at 37°C in 5% carbon dioxide. After incubating the ARPE-19 and R-28 cells for 24 h to reach confluence, the media in the wells were replaced with the respective serum-free media containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA). These cells were then incubated for another 24 h in these serum-free media to render them quiescent (non-proliferating). This step was omitted for HMVEC as they did not tolerate additional incubation in serum-free media for 24 h (even untreated controls had very low viability). Also, a stable (non-proliferating) population of HMVEC was achieved even without additional incubation. Thus, for the HMVEC cultures, the HQ was added without the additional 24-h incubation in serum-free media.

Exposure to hydroquinone

HQ commercially available as a powder was solubilized in dimethyl sulfoxide (DMSO). Cells were treated with 500 μM, 200 μM, 100 μM, 50 μM concentrations of HQ for 24 h. ARPE-19, R-28, HMVEC cultures were also treated with equivalent amount of DMSO as control cultures.

Cell viability assay

Cell viability assay was performed as described by Narayanan *et al*.[15] Briefly, cells were harvested from the 35-mm dishes by treatment with 0.2% trypsin-EDTA and then incubated at 37°C for 5 minutes. The cells were centrifuged at 1000 rpm for 5 minutes and then resuspended in 1 ml of culture medium. Automated cell viability analysis was performed (Vi Cell analyzer; Beckman Coulter Inc., Fullerton, CA, USA). The analyzer performs an automated trypan blue dye-exclusion assay and gives the percentage viability of cells.

Caspase detection

Caspase-3/7 activities were detected with the use of detection kits (Carboxyfluorescein FLICA Apoptosis Detection kits; Immunochemistry Technologies LLC, Bloomington, MN, USA). The FLICA reagent has an optimal excitation range from 488 to 492 nm and an emission range from 515 to 535 nm. Apoptosis was quantified as the level of fluorescence emitted from FLICA probes bound to caspases.

At the designated time period, the wells were rinsed briefly with fresh culture media, replaced with 300 μl/well of 1× FLICA solution in culture media, and incubated at 37°C for 1 h under 5% CO_2. Cells were washed with phosphate-buffered saline (PBS). The following controls were included: untreated ARPE-19, R-28, HMVEC without FLICA to exclude autofluorescence from cells; untreated ARPE-19, R-28, HMVEC with FLICA for comparison of caspase activity of treated cells; tissue culture plate wells without cells with buffer alone to represent the background levels; tissue culture plate wells without cells with culture media + DMSO to exclude cross-reaction of FLICA with DMSO + culture media; ARPE-19, R-28, HMVEC with DMSO and FLICA to account for any cross-fluorescence between untreated cells and DMSO.

Quantitative calculations of caspase activities were performed with a fluorescence image scanning unit instrument (FMBIO III; Hitachi, Yokohama, Japan). The caspase activity was measured as average signal intensity of the fluorescence of the pixels in a designated spot – Mean Signal Intensity (msi).

DNA fragmentation

The QIAamp DNA Mini Kit (QIAGEN Sample and Assay Technologies, Valencia, CA, USA) was used to extract the DNA from the HQ-treated cell cultures. The Nanodrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA) was used to determine the DNA content of each sample. For the assessment of DNA fragmentation, 2.5 μg of DNA was added to each lane of a 3% agarose gel and electrophoresed for 5 h at 80 V. The gel was stained with 5% ethidium bromide and visualized and photographed with fluorescence image scanning instrument (FMBIO III; Hitachi).

Lactate dehydrogenase cytotoxicity assay

The LDH Cytotoxicity Assay kit II (BioVision, Inc., Mountain View, CA, USA) was used to measure the lactate dehydrogenase (LDH) enzyme released from the cells as an index of necrosis or nonapoptotic cell death. The assay was performed as per the manufacturer's protocol. LDH Cytotoxicity Assay kit II utilizes the water soluble tetrazolium salt (WST) reagent for detection of LDH released from the damaged cells. The assay uses an enzyme coupling reaction; LDH oxidizes lactate to generate NADH which then reacts with WST to generate a yellow color. LDH activity was then quantified with a spectrophotometer (Perkin Elmer, Inc., IL) at 450 nm optical density (OD). The data presented were from three different sets of experiments. Each concentration was performed in triplicate and data were plotted according to manufacturer's protocol.

Statistical analysis

Data were subjected to statistical analysis by analysis of variance (ANOVA) (Prism, ver. 3.0; GraphPad Software Inc., San Diego, CA, USA). Newman–Keuls multiple-comparison test was done to compare the data within each experiment. $P < 0.05$ was considered statistically significant. Error bars in the graphs represent Standard Error of Mean (SEM) with experiments performed in triplicate.

Results

Cell viability studies [Fig. 1]

ARPE-19 cells showed a significant decrease in cell viability after exposure to HQ for 24 h. Untreated and DMSO-treated equivalent cultures of 500 μM showed cell viabilities of 96.10 ± 1.84% and 96.25 ± 0.78%, respectively. Cell viabilities were 26.60 ± 1.56% ($P < 0.001$), 33.50 ± 3.53% ($P < 0.001$) and 52.95 ± 2.05% ($P < 0.001$) at doses of 500 μM, 200 μM, and 100 μM HQ, respectively. At the lower concentrations of 50 μM HQ, cell viability was 95.70 ± 0.42 ($P > 0.05$).

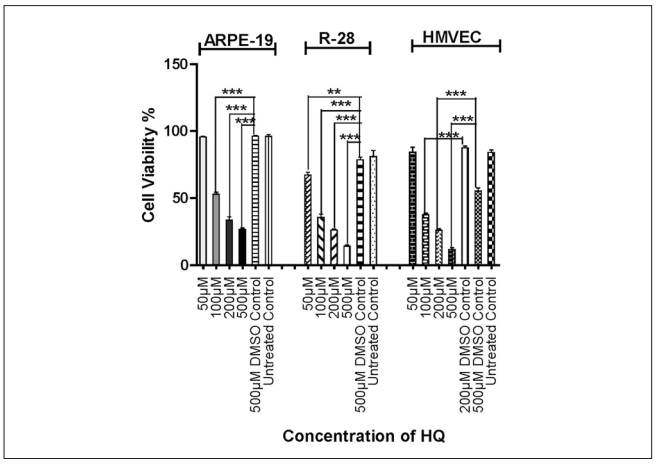

Figure 1: Cell viability of cultured cells (ARPE-19, R-28, HMVEC) following HQ treatment for 24 h. Representative control and HQ-treated samples are presented for each cell line. DMSO: Dimethyl sulfoxide. ***Statistically significant ($P < 0.001$); **statistically significant ($P < 0.01$)

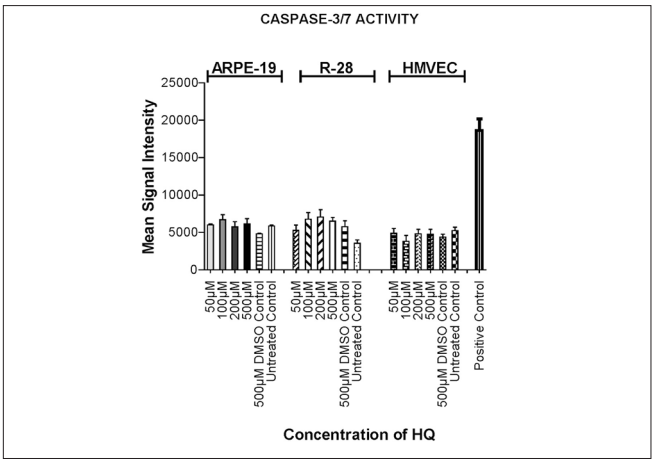

Figure 2: All three cell lines (ARPE-19, R-28, HMVEC) did not show increase in caspase-3/7 activity following 24-h exposure of HQ. Mean signal intensity (msi) is presented for representative control and HQ-treated samples

R-28 cells showed a significant decrease in cell viability after exposure to HQ for 24 h. Untreated and DMSO-treated equivalent culture of 500 µM showed cell viabilities of 81.050 ± 6.43% and 78.56 ± 2.90%, respectively. Cell viabilities were 14.15 ± 1.20% ($P < 0.001$), 26.20 ± 1.13% ($P < 0.001$), and 35.70 ± 3.25% ($P < 0.001$) at doses of 500 µM, 200 µM, and 100 µM HQ, respectively. At the lower concentrations of 50 µM HQ, cell viability was 67.25 ± 2.89% ($P < 0.01$).

HMVEC cultures showed a significant decrease in cell viability after exposure to HQ for 24 h. Untreated cell culture showed cell viability of 84.00 ± 3.59%. DMSO-treated equivalent culture of 500 µM and 200 µM showed cell viabilities of 55.37 ± 3.76% and 87.50 ± 2.19%, respectively. Cell viabilities were 11.73 ± 2.27% ($P < 0.001$), 25.93 ± 2.17% ($P < 0.001$), and 37.52 ± 1.93% ($P < 0.001$) at doses of 500 µM, 200 µM, and 100 µM HQ, respectively. At the lower concentrations of 50 µM HQ, cell viability was 84.20 ± 6.78% ($P > 0.05$).

Caspase-3/7 activity [Fig. 2]

ARPE-19 cells treated with 500 µM, 200 µM, 100 µM, and 50 µM of HQ showed msi of 6132.27 ± 1011.5 ($P > 0.05$), 5733.83 ± 1014.3 ($P > 0.05$), 6721.74 ± 901.6 ($P > 0.05$), and 6015.19 ± 208.6 ($P > 0.05$), respectively. Values for untreated cells and DMSO-equivalent cultures 500 µM of HQ were 5861.70 ± 220.7 and 4820.83 ± 83.3, respectively.

R-28 cells treated with 500 µM, 200 µM, 100 µM, and 50 µM of HQ showed msi of 6514.40 ± 692.1 ($P > 0.05$), 7074.35 ± 1357.8 ($P > 0.05$), 6777.15 ± 1253.2 ($P > 0.05$), and 5280.05 ± 1008.4 ($P > 0.05$), respectively. Values for untreated cells and DMSO-equivalent cultures 500 µM of HQ were 3580.00 ± 595.3 and 5783.90 ± 1073.5, respectively.

HMVEC cultures cells treated with 500 µM, 200 µM, 100 µM, and 50 µM of HQ showed msi of 4762.00 ± 1153.7 ($P > 0.05$), 4831.33 ± 1001.4 ($P > 0.05$), 3813.40 ± 1339.8 ($P > 0.05$), and 4887.91 ± 1098.3 ($P > 0.05$), respectively. Values for untreated cells and DMSO-equivalent cultures 500 µM of HQ were 5262.45 ± 793.0 and 4376.02 ± 709.4, respectively.

DNA fragmentation [Fig. 3]

None of the cell lines showed any 200 bp DNA fragmentation pattern on 3% agarose gels.

Lactate dehydrogenase release assay [Fig. 4]

ARPE-19 cells showed an increase in LDH levels after exposure to HQ for 24 h. Untreated and DMSO-treated equivalent culture of 500 µM showed LDH levels of 18.36 ± 1.37% and 18.83 ± 1.74%, respectively. LDH values were 53.56 ± 2.61% ($P < 0.001$), 48.30 ± 7.25% ($P < 0.001$), and 41.13 ± 2.05% ($P < 0.001$) at doses of 500 µM, 200 µM, and 100 µM HQ, respectively. At the lower concentrations of 50 µM HQ, the LDH value was 19.76 ± 4.35% ($P > 0.05$).

R-28 cells showed an increase in LDH value after exposure to HQ for 24 h. Untreated and DMSO-treated equivalent culture of 500 µM showed LDH levels of 20.36 ± 2.12% and 18.46 ± 3.73%, respectively. LDH values were 53.23 ± 6.10% ($P < 0.001$), 46.63 ± 8.76% ($P < 0.001$), 44.09 ± 5.29% ($P < 0.001$), and 41.96 ± 2.82% ($P > 0.001$) at doses of 500 µM, 200 µM, 100 µM, and 50 µM HQ, respectively.

HMVEC cultures showed an increase in LDH value after exposure to HQ for 24 h. Untreated and DMSO-treated equivalent culture of 500 µM showed cell viabilities of 20.37 ± 2.12% and 18.47 ± 3.73%, respectively. LDH values were 45.26 ± 6.83% ($P < 0.001$), 42.30 ± 5.25% ($P < 0.001$), and 37.16 ± 1.90% ($P < 0.001$) at doses of 500 µM, 200 µM, and 100 µM HQ, respectively. At the lower concentrations of 50 µM HQ, the LDH value was 17.63 ± 4.23% ($P > 0.05$).

Discussion

The present study demonstrated that after 24-h treatment with HQ, the ARPE-19 cells showed no loss of cell viability at 50 µM HQ but exhibited significant cell loss at doses greater than 100 µM. This agrees with the report of Chen *et al*. who found necrotic cell death of bone marrow mononuclear cells at concentrations above 50 µM.[16] Our findings differ from those of Strunnikova *et al*. who found no loss of RPE cell viability at 100 µM HQ after 16 h of incubation.[17] However, our results

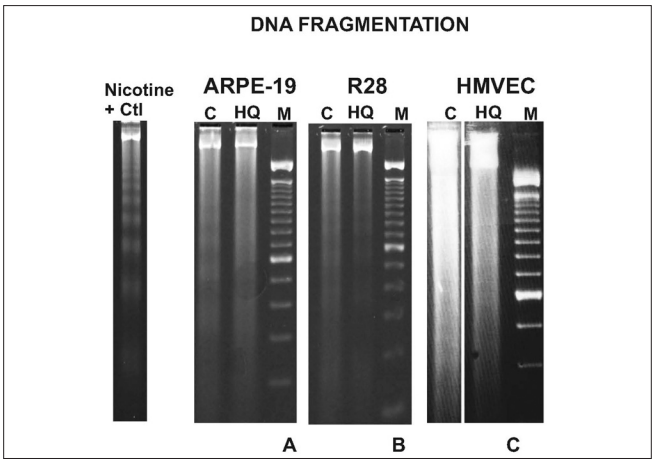

Figure 3: Lack of DNA fragmentation following HQ treatment of cultured cells. Representative control and HQ-treated samples are presented along with molecular weight markers. The electrophoretic patterns are consistent with non-apoptotic cell death. A, ARPE-19 cells; B, R-28 cells; C, HMVEC cultures

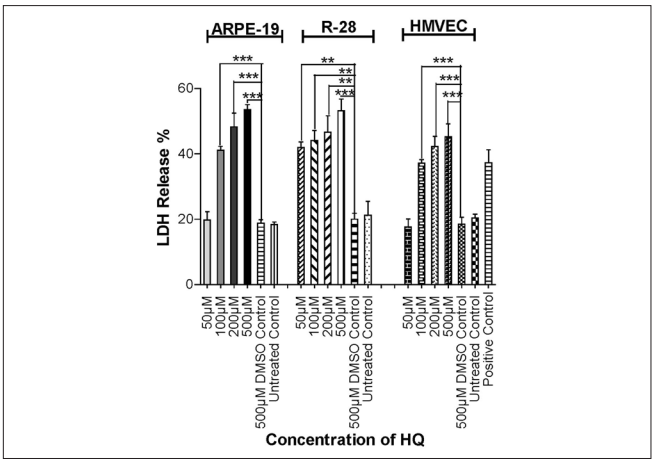

Figure 4: LDH release from the cultured cells (ARPE-19, R-28, HMVEC) following HQ treatment for 24 h. Representative control and HQ-treated samples are presented for each cell line. DMSO: Dimethyl sulfoxide. ***Statistically significant ($P < 0.001$); **statistically significant ($P < 0.01$)

are similar to those of Marin-Castaño *et al*. who found RPE cell damage at 250–400 µM HQ.[18] A recent study showed the formation of sub-RPE deposits and diffuse thickening of Bruch's membrane in mice after exposing them to cigarette smoke or HQ.[19] This is significant because histochemical studies reported the presence of extracellular protein and/or lipid deposits (drusen) between the basal lamina of the RPE cells and the inner layer of the Bruch's membrane carries an increased risk of progressing to an advanced form of AMD, either geographic atrophy (dry AMD) or choroidal neovascularization (wet AMD).[20] Cigarette smoke contains many toxicants, including acrolein that damages mitochondrial function.[21] It is becoming apparent that different cigarette smoke components may have different mechanisms of action on the human RPE cells. In a recent study, we have found elevation of caspase-3/7, caspase-8, caspase-9, caspase-12 activities in ARPE-19 cells *in vitro* with benzo(e)pyrene (B(e)P), a polycyclic aromatic hydrocarbon (PAH) present in cigarette smoke,[22] while HQ did not elevate caspase-3/7 activity in this study.

R-28 cells had significant loss of cell viability at 50 µM HQ in addition to the higher doses (100 µM, 200 µM, 500 µM), indicating that the R-28 cells were more sensitive to HQ than the ARPE-19 cells. This increased sensitivity to chemicals by R-28 cells has also been reported in cultures treated with 7-ketocholesterol[23] and trypan blue.[15] Our findings are consistent with those of Haenen *et al*. who found loss of cell viability and LDH release in renal proximal tubular cells after 250 µM and 500 µM HQ treatment.[24] Our findings differ from those of Terasaka el al. who reported caspase-3 and caspase-9 activation at 50 µM HQ in HL60 cells after 6 h of incubation.[25] Photoreceptor inner segments have a high content of mitochondria and unsaturated fatty acids, which makes these cells potential targets of oxidative injury (i.e. blue light, HQ) via apoptotic or non-apoptotic pathways.[26]

HMVEC cultures showed significant loss of cell viability at HQ doses 100 µM, 200 µM, and 500 µM, but not at the lower dose of 50 µM HQ. Smith *et al*. found loss of cell viability in a dose-dependent manner starting from 2 µM HQ in human CD34-positive blood progenitor cells.[27] Hiraku *et al*. reported necrotic cell death at 100 µM HQ and higher concentrations.[28] These findings differ from those of Dong-Xiao Shen *et al*. who found apoptotic cell death at 100 µM HQ in human embryonic kidney cells (HEK293).[29] Although RPE is the primary target of HQ,[19] it can affect other cells in the outer retina, such as photoreceptor cells and choriocapillaries endothelium. Sub-RPE deposit formation could be the result of an initial insult to the endothelium,[30] which could contribute to the formation of sub-RPE deposits.

We realize that there are some limitations of this study. The HQ concentrations used cannot be extrapolated directly to clinical practice, as this study was performed *in vitro*. In addition, the level of HQ in the retina of smokers is unknown. However, there are 40% higher levels of HQ in smoker's blood compared to that of non-smoker.[31] Therefore, it is not unreasonable to expect that the retina and RPE cells are exposed to higher HQ levels in heavy smokers. Another drawback of our study is that the cell cultures were still capable of some proliferation, which is different from the normal clinical condition and may limit the interpretation of the results. However, we believe the data presented are significant in light of the present clinical treatment approaches of AMD.

To the best of our knowledge, this is the first study showing non-apoptotic damage in all three cell types representative of the retina after exposure to the cigarette smoke toxicant, HQ. Understanding the AMD pathogenesis could be helpful for drug development targeting the specific pathways involved. As different groups of compounds have different mechanisms of cell death, to protect the cells from these noxious effects, we need to develop a treatment that can antagonize a variety of pathways. Furthermore, it demonstrates that the ARPE-19, R-28, and HMVEC culture systems can be used to generate information of the biochemical mechanisms of chemical toxicants and protective agents.

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