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2-ethylpyridine, a cigarette smoke component, causes mitochondrial damage in human retinal pigment epithelial cells *in vitro*

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Abstract

Purpose:

Our goal was to identify the cellular and molecular effects of 2-ethylpyridine (2-EP, a component of cigarette smoke) on human retinal pigment epithelial cells (ARPE-19) *in vitro*.

Materials and Methods:

ARPE-19 cells were exposed to varying concentrations of 2-EP. Cell viability (CV) was measured by a trypan blue dye exclusion assay. Caspase-3/7 and caspase-9 activities were measured by fluorochrome assays. The production of reactive oxygen/nitrogen species (ROS/RNS) was detected with a 2',7'-dichlorodihydrofluorescein diacetate dye assay. The JC-1 assay was used to measure mitochondrial membrane potential ($\Delta\Psi m$). Mitochondrial redox potential was measured using a RedoxSensor Red kit and mitochondria were evaluated with Mitotracker dye.

Results:

After 2-EP exposure, ARPE-19 cells showed significantly decreased CV, increased caspase-3/7 and caspase-9 activities, elevated ROS/RNS levels, decreased $\Delta\Psi m$ value and decreased redox fluorescence when compared with control samples.

Conclusions:

These results show that 2-EP treatment induced cell death by caspase-dependent apoptosis associated with an oxidative stress and mitochondrial dysfunction. These data represent a possible mechanism by which smoking contributes to age-related macular degeneration and other retinal diseases and identify mitochondria as a target for future therapeutic interventions.

Keywords: 2-ethylpyridine, apoptosis, ARPE-19 cells, cigarette smoke toxicant, mitochondrial membrane

potential, reactive oxygen/nitrogen species

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in individuals over 55 years of age and the prevalence is predicted to increase in the aging population.[1,2] There are different forms of AMD, one being the "wet" or neovascular form and the other being "dry" or atrophic AMD. In wet AMD, choroidal neovascularization develops, causing hemorrhage, swelling and macular scarring, resulting in severe vision loss. Dry AMD is characterized as atrophy of photoreceptors and retinal pigment epithelium (RPE), which can result in a decreased central vision over time.[3] The clinical features of AMD are well-documented but, the mechanisms for the pathogenesis of AMD are still unclear.[4]

Risk-factors for AMD include smoking, higher body mass index, nutrition and genetics.[5] Among these, cigarette smoking is one of the strongest factors associated with developing the most severe forms of AMD.[6,7] Current smokers have a 45% chance of developing early AMD and exhibit enhanced disease progression compared with non-smokers.[8] The degeneration of RPE cells and abnormalities in Bruch's membrane are the earliest stages of AMD[9,10] while photoreceptor damage, especially within the macular region, is considered to be a later stage of AMD pathology.[11] *In vitro* studies show that cigarette smoke extract can induce human RPE cell death and alterations in extracellular matrix synthesis.[12] Therefore, the survival of RPE cells in smokers has been a major area of study.

Pyridines are predominant constituents of cigarette smoke[13,14] and one of its most toxic derivatives is 2-ethylpyridine (2-EP). The pyridine derivatives can be toxic to cells in several ways. For example, they depress the antioxidant activity of α-tocopherol,[15] catalyze the oxidation of lard and other fats,[16] inhibit oocyte maturation and sperm capacitation[17] and generate fatty acid peroxides that disrupt vascular permeability and poison enzyme systems.[18] Biochemically, while pyridine itself is not strongly toxic to cells, the addition of methyl or ethyl groups increases its toxicity significantly.[13,14] The derivative 2-EP has ethyl groups and reportedly inhibits the growth of chick chorioallantoic membranes,[14] blocks hamster (Mesocricetus auratus) oviduct functioning[19] and alters the growth and survival of cultured mammalian cells.[20]

The present study is the first to demonstrate that 2-EP exposure increases caspase activities, oxidative stress and mitochondrial dysfunction in human RPE cells *in vitro*. Our data support our hypothesis that 2-EP, a constituent from cigarette smoke, may play a significant role in promoting the onset and progression of AMD by damaging the mitochondria within the RPE cells.

Materials and Methods

Cell culture and treatments

ARPE-19 cells (ATCC, Manassa, VA) were grown in a 1:1 mixture (vol/vol) of Dulbecco's modified Eagle's and Ham's nutrient mixture F-12 media (Invitrogen-Gibco, Carlsbad, CA), 10 mM 1x nonessential amino acids, 0.37% sodium bicarbonate, 0.058% L-glutamine, 10% fetal bovine serum (FBS) and antibiotics (penicillin G 100 U/ml, streptomycin sulfate 0.1 mg/ml, gentamicin 10 μg/ml, amphotericin-B 2.5 μg/ml). The ARPE-19 cells are a human diploid cell line that show structural and functional properties similar to RPE cells *in vivo*[21] and are commonly used in retinal research.[22,23,24] All procedures followed were in accordance with the ethical standards committee on human experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 2000 (available http://www.wma.net/e/policy/17-c_e.html).

The ARPE-19 cells used for these experiments were derived from passages 17 to 20 and incubated at 37°C in 5% CO_2. Cells were plated in either 6, 24, or 96 well plates (Becton Dickinson Labware, Franklin Lakes, NJ) for the assays to measure cell viability (CV) (5×10^5 cells/well), caspase-3/7 and caspase-9 activities (1.2×10^5 cells/well), mitochondrial membrane potential (1.2×10^5 cells/well), reactive oxygen/nitrogen species (ROS/RNS)

production (1.2×10^5 cells/well), redox potential (1.0×10^5 cells/well) and mitochondrial densities (1.0×10^5 cells/well). We have found that ARPE-19 cells are very sensitive to rapid decreases in levels of serum, so the cells were initially cultured in 10% FBS, but once established, the media were changed to 2%FBS for 6 h, which makes them relatively non-proliferating.[25] Cells were then incubated for 24 h in serum free culture media with or without 2-EP.

Exposure to 2-EP

2-EP was purchased from Sigma Aldrich Inc., St. Louis, MO and the stock 100 mM solution was prepared in 1 ml of deionized distilled water. Thereafter, 40 μM 2-EP was prepared by adding 4 μl from the 100 mM 2-EP stock solution to 10 mL of culture media. Cells were treated with 40 μM, 30 μM, 20 μM and 10 μM 2-EP for 24 h. Some cells were incubated without 2-EP and served as control cultures.

CV studies

The CV assay was performed as described by Narayanan *et al.*[26] Briefly, cells were harvested from the 6 well plates by incubation at 37°C for 5 minutes with 0.2% trypsin-ethylenediaminetetracetic acid. Cells were centrifuged at 1000 rpm for 5 min and resuspended in 1 ml of culture medium. CV was analyzed with a Vi-Cell automated analyzer (Beckman Coulter Inc., Fullerton, CA), which performs an automated trypan blue dye-exclusion assay and gives the percentage of viable cells.

Caspase-3/7 and caspase-9 activities

Caspase-3/7 and caspase-9 activities were detected using Carboxyfluorescein FLICA Apoptosis Detection Kits (Immunochemistry Technologies LLC, Bloomington, MN). 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. Non-apoptotic cells appeared unstained, whereas, cells undergoing apoptosis fluoresced brightly.

At the designated time periods, the wells were rinsed briefly with fresh culture media, replaced with 300 μl/well of 1x FLICA solution in culture media and incubated at 37°C for 1 h in 5% CO_2. Cells were washed with phosphate-buffered saline (PBS). The following controls were included: Untreated ARPE-19 cells without FLICA to exclude auto-fluorescence from ARPE-19 cells; untreated ARPE-19 cells with FLICA for comparison of caspase activity of treated cells; and tissue culture plate wells without cells with buffer alone to represent the background level. Quantitative calculations of caspase activities were performed with a fluorescence image scanner (FMBIO III; Hitachi, Yokohama, Japan). Caspase activities were measured as mean signal intensity (msi) of the fluorescence of the pixels in a designated spot.

Deoxyribonucleic acid fragmentation assay

ARPE-19 cells (5×10^5 cells/well) were plated overnight in 6-well plates and then incubated for another 24 h with different concentrations of 2-EP. DNA was extracted using a QIAamp DNA Micro-kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were separated by electrophoresis on 3% agarose gels and stained with 5% ethidium bromide. A 100 base pair (bp) marker was used and images were captured with a fluorescence imaging scanner (FMBIO III; Hitachi).

ROS/RNS levels

ROS/RNS production was measured with the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate assay (H_2 DCFDA; Invitrogen-Gibco)[27] which detects hydrogen peroxide, peroxyl radicals and peroxynitrite anions. Cells were washed with a sterile PBS, incubated with 500 μl of 10 μM H_2 DCFDA for 30 min at 37°C and again washed with PBS. The H_2 DCFDA (10 μM) was prepared by adding 2 μl of 5 mM (H_2 DCFDA) stock/ml in serum free culture media. 5 mM H_2 DCFDA stock solution was prepared fresh by mixing 0.005 g of H_2 DCFDA

in 2.05 ml of DMSO. ROS/RNS production was measured with the scanning unit (excitation 488 nm, emission 520 nm, FMBIO III; Hitachi).

Mitochondrial membrane potential (ΔΨm) assay

Loss of ΔΨm, a hallmark for early apoptosis, was measured using the JC-1 mitochondrial membrane potential detection kit (Biotium, Hayward, CA). JC-1 contains a cationic dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl- carbocyanine-iodide) that fluoresces red within the mitochondria of alive cells. In the dying cells, the mitochondrial membrane potential collapses and the cationic dye remains in the cytoplasm but fluoresces green. In healthy cells, the red to green fluorescence ratio is higher compared to the cells entering apoptosis.

We performed the JC-1 assay as per the supplier's instructions. Briefly, after 24 h 2-EP exposure, the cells were rinsed with fresh media and incubated for 15 min with 500 µl/well of JC-1 reagent in culture media. Images were captured using a fluorescence image scanner (FMBIO III; Hitachi) and the red/green fluorescence ratios were calculated.

Measurements for redox potential and mitochondria levels

We used RedoxSensor Red CC-1, a non-fluorescent dye, to assess 2-EP induced mitochondrial-related oxidative stress. RedoxSensor Red enters live cells through a non-endocytic pathway and in the case of non-oxidative cytosolic conditions accumulates in the mitochondria. However, in the presence of oxidants in the cytosol, the dye gets oxidized to a red-fluorescent product and instead accumulates in the cytosol due to the altered mitochondrial membrane potential.[28] The selective distributions of the oxidized dye between mitochondria and lysosomes are the fundamental basis of this assay. Cells with disrupted mitochondrial membranes have lower accumulations of oxidized RedoxSensor Red than intact mitochondria and hence have diminished fluorescence.[28] The fluorescent intensity of oxidized RedoxSensor Red within the mitochondria can be quantified with MitoTracker Green, which is a fluorescent stain that preferentially accumulates inside the mitochondria regardless of mitochondrial potential.[29,30]

This redox-dependent oxidative assay was a modification of the supplier's instructions.[28] Briefly, confluent ARPE-19 cells were exposed to 2-EP (40, 30, 20 and 10 µM) for 24 h. Then, 5 µM of RedoxSensor Red and 1 µM of MitoTracker Green (Molecular Probes, Eugene, OR) were added to each dish and incubated for 10 min at 37°C. Cells were washed twice with PBS. In each well the red (580 nm) and green (520 nm) fluorescence were quantified using a scanning unit (FMBIO, Hitachi).[31]

Statistical analysis

Data were subjected to statistical analysis by ANOVA (Prism, version 3.0; GraphPad Software Inc., San Diego, CA). The Newman-Keuls multiple-comparison test was performed to compare the data within each experiment. A $P < 0.05$ was considered statistically significant. Error bars in the graphs represent standard error mean. Experiments were repeated 3 times. For experimental and control samples, the treatments were run in triplicate and the values were combined for the final data.

Results

CV Studies

ARPE-19 cells treated for 24 h with 2-EP at 20 µM, 30 µM and 40 µM concentrations showed significant decreases in CV compared to untreated cultures [Fig. 1]. The mean percent of viable cells was 67.5 ± 2.5 ($P < 0.001$), 56 ± 2 ($P < 0.001$) and 39.3 ± 3.3 ($P < 0.001$) for 2-EP at 20 µM, 30 µM and 40 µM, respectively as compared to untreated cultures (98.3 ± 1.1). After exposure to 10 µM, the mean CV was not significantly decreased (90.8 ± 1.2) as compared to untreated control ($P > 0.05$).

Caspase-3/7 activity

Caspase-3/7 activity is the hallmark and final common pathway of apoptosis. In ARPE-19 cells, caspase-3/7 activities increased significantly after treatment for 24 h with 2-EP at 20 μM, 30 μM and 40 μM as compared to untreated control [Fig. 2a]. The mean fluorescence values were 22,061 ± 812 msi ($P < 0.001$), 25,529 ± 290 msi ($P < 0.001$) and 29,666 ± 1201 msi ($P < 0.001$) for 2-EP at 20 μM, 30 μM and 40 μM, respectively, as compared to the untreated cultures (3666 ± 240 msi). Cells treated with 10 μM 2-EP did not show a significant increase in caspase-3/7 activity (4466 ± 290 msi, $P > 0.05$) as compared to untreated cultures. To confirm that apoptosis occurred, analyses of DNA fragmentation patterns were performed. The DNA from the untreated control cultures remained intact at the top of the gel [Fig. 2b, Lane 1]. The 2EP-treated samples showed DNA bands that laddered in approximately 200 bp increments, consistent with apoptosis (lane 2). Lane 3 shows the marker with 100 bp interval bands.

Caspase-9 activity

The ARPE-19 cells treated for 24 h with 2-EP had increased caspase-9 activity as compared to untreated control cultures [Fig. 3]. The mean fluorescence values for 2-EP at 20 μM (17,000 ± 577 msi, $P < 0.001$), 30 μM (24,333 ± 1201 msi, $P < 0.001$) and 40 μM (26,000 ± 1154 msi, $P < 0.001$) were significantly elevated as compared to the untreated cultures (2200 ± 230 msi). Cells treated with 2-EP at 10 μM concentration did not show a significant increase in caspase-9 activity (3900 ± 493 msi, $P > 0.05$).

ROS/RNS levels

ARPE-19 cells treated for 24 h with 2-EP showed significantly increased ROS/RNS levels as compared to untreated cultures [Fig. 4a]. The mean fluorescence values were 16,500 ± 500 msi ($P < 0.01$), 22,750 ± 750 msi ($P < 0.001$) and 28,600 ± 601 msi ($P < 0.001$) for 2-EP at 20 μM, 30 μM and 40 μM, respectively, as compared to untreated control cultures (3505 ± 500.3 msi). Cells treated at 10 μM 2-EP did not show significantly elevated ROS/RNS values (4700 ± 300 msi, $P > 0.05$) as compared to untreated cultures.

Mitochondrial membrane potential (ΔΨm) assay

ARPE-19 cells treated for 24 h with 2-EP showed significantly decreased ΔΨm as compared to untreated culture [Fig. 4b]. The mean ΔΨm values were 4.03 ± 0.09 ($P < 0.001$), 3.29 ± 0.26 ($P < 0.001$) and 1.87 ± 0.06 ($P < 0.001$) for 2-EP at 20 μM, 30 μM and 40 μM, respectively, as compared to untreated control culture (6.95 ± 0.09). Cells treated at 10 μM 2-EP concentration did not show significantly decreased ΔΨm value (6.4 ± 0.15, $P > 0.05$) as compared to untreated cultures.

Redox potential and levels of mitochondria

ARPE-19 cells treated 24 h with 2-EP at 20 μM, 30 μM and 40 μM showed significant loss of redox potential compared to untreated culture [Fig. 5a]. The mean fluorescence values were 1566 ± 120 msi ($P < 0.001$), 1133 ± 88 msi ($P < 0.001$) and 1033 ± 60 msi ($P < 0.001$) for 2-EP at 20 μM, 30 μM and 40 μM, respectively, as compared to untreated culture (3383 ± 130 msi). Cells treated by 2-EP at 10 μM had a similar fluorescence value (3033 ± 145 msi, $P > 0.05$) as untreated cultures.

The loss of redox potential can be due to the damage of mitochondria or cellular lysosomes. One method is used to distinguish between the two mechanisms is to analyze for the number of mitochondria within the cells using the mitochondrial-specific MitoTracker Green dye. If the mitochondria-specific staining decreases parallel the redox potential values, it suggests that the decline is associated with mitochondrial damage and loss. We found that the ARPE-19 cells treated for 24 h with 2-EP showed significantly decreased Mitotracker fluorescence as compared to untreated culture [Fig. 5b]. At 20 μM 2-EP, the mean fluorescence value was 4900 ± 264 msi ($P < 0.01$), at 30 μM 2-EP the value was 2966 ± 176 msi ($P < 0.01$) and at 40 μM 2-EP the value was 1966 ± 145 msi ($P <$

0.001) compared to untreated culture (8233 ± 272 msi). Cells treated by 2-EP at 10 μM concentration showed a similar fluorescence value (7533 ± 272 msi, *P*> 0.05) as untreated cultures.

Discussion

There are several derivatives of pyridines in cigarette smoke and their toxic potential varies according to the functional group and position of substitution.[14,19] 2-EP with its ethyl group substituted at the second carbon position of the pyridine ring, is far more potent than the parent molecule[13] and it is present in the range of 0.66-0.76 μg/cigarette.[32] One limitation of our work is that it is difficult to determine the concentrations of 2-EP within the blood and retina tissues because of the variability in smoking devices (cigarettes of various sizes, cigars and tobacco pipes), the history and frequency of smoking and the average inhalation capacity of the smoker. Nevertheless, it is well accepted that the cumulative effects of cigarette toxicants over time cause cell death and increase susceptibility to oxidative damage.[33] In addition, there is a well-recognized relationship between pack-years of smoking and neovascular AMD, which follows a dose-response effect.[34]

There are cross-sectional, case-control and prospective studies which consistently show strong associations between AMD and cigarette smoking.[6,35,36,37,38,39] The interpretation from our results is limited because it is an *in vitro* system and uses only a single cigarette smoke component, 2-EP. However, our study clearly shows that in response to 2-EP, ARPE-19 cells undergo apoptosis, as reflected by increased caspase-3/7 and capase-9 activities and DNA laddering, which is similar to the response seen in RPE cells after exposure to B (e) P, another cigarette smoke element.[25] Using a different cell type it has been shown that R28 cells undergo apoptosis at lower B (e) *P*doses and necrosis at higher doses of B (e) P.[40] In contrast, nicotine-treated R28 cells show damage through non-caspase, non-calpain mediated pathways and the toxicity generated by Benzo (e) Pyrene (B (e) P) in human microvascular endothelial cells occurs via necrosis.[40] Still other studies demonstrate that exposure of RPE cells to the cigarette smoke extracts of hydroquinone or acrolein can cause oxidative damage and apoptosis.[41,42,43] In retinal Móller cells treated with catechol, both apoptosis and oxidative stress involving mitochondrial dysfunction occur.[44] Finally, treatment of retinal and vascular cells with hydroquinone show that the mechanism of cell death is through non-apoptotic pathway[23] and that factors from proinflammatory pathways are induced.[45] The combination of studies shows that when it comes to cigarette-related damage, there is variability depending upon the cell types, toxicant exposure concentrations and mechanisms of cell death. This is significant because if effective protective drugs are to be developed, all known targets and pathways must be identified.

Mitochondria are major sites for energy production so their integrity is very important for cell survival, yet multiple assays in our study showed that significant mitochondrial damage occurs after 2-EP exposure. The 2-EP-treated ARPE-19 cells had increased ROS/RNS levels, which indicate that oxidative stress plays an important role in its cytotoxicity. ROS production may inflict further damage to the mitochondria, thereby leading to vicious cycle (s) of ROS-related damage within cells.[46] Earlier work has shown that numerous mitochondria molecules are vulnerable to ROS damage, including proteins, lipids and the mitochondrial DNA (mtDNA). Interestingly, studies show that mtDNA is particularly susceptible to injury from oxidation, in part because it lacks histones or good mechanisms to repair damaged mtDNA.[47] The mtDNA has 37 genes that encode 13 proteins critical for oxidative phosphorylation (OXPHOS). As the mtDNA is damaged, the OXPHOS becomes less efficient and there is more leakage of electrons through the electron transport chain, with a resultant increase of endogenous ROS being generated from the mitochondria. As this vicious cycle continues, there are higher numbers of deletions and/or rearrangements within the mtDNA, such as found in AMD retinas,[48,49] which in turn can again lower energy production levels.[50]

Caspase-9 is the mitochondrial-associated member in the caspase-cascade pathway and its activation represents important steps in numerous mitochondrion-related apoptotic processes.[51] We found increased caspase-9 activity in 2-EP-treated cells, which is consistent with studies of ARPE-19 cells showing caspase-9 activation after

treatment with B (e) P.[25] 2-EP-treated cells also showed significantly decreased mitochondrial membrane potential (ΔΨm) and reduced redox level, all which are signs for mitochondrial-related toxicity. By using MitoTracker Green dye, we showed a parallel decrease of redox potential and mitochondrial numbers, suggesting that the decline in redox was mitochondrial-damage rather than due to damage of the lysosomes within the cells. Our findings reinforce the importance of finding mitochondrial-protecting drugs to inhibit mitochondrial damage caused by harmful tobacco components.

Decreased mitochondrial membrane potential (ΔΨm) is an early sign of apoptosis.[52,53] Under normal physiological conditions, the mitochondrial permeability transition (MPT) pore, which is situated across the inner and outer membrane, allows for limited diffusion of ions into the mitochondria. Mitochondrial toxins alter the function of the MPT pore, leading to its prolonged opening, rapid movement of ions through the pore and extensive mitochondrial swelling that results in cell death.[54] Loss of the ΔΨm after 2-EP treatment shows that this drug induces early stages of mitochondrial damage, intermediate stages with activation of the mitochondrial caspase pathway (caspase-9) and also the late stage of apoptosis with effector caspase-3/7 activation.

Conclusion

Our findings show that 2-EP can cause mitochondrial dysfunction in human retinal cells, leading to loss of the mitochondrial membrane potential, activation of mitochondrial-specific capsase-9, decreased redox potential and finally increased apoptosis. Our findings are significant because they show a connection between 2-EP related oxidative stress and mitochondrial damage and demonstrate that mitochondria are excellent targets for drug therapies designed to protect the stressed human retinal cells. Our future studies will focus on identification of effective inhibitors or reversal agents for 2-EP and other smoking-related compounds. This approach may benefit not only AMD patients, which are high risk due to their smoking habits, but also individuals that have other cigarette smoke-related diseases.

Footnotes

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Conflict of Interest: None declared.

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Figures and Tables

Figure 1

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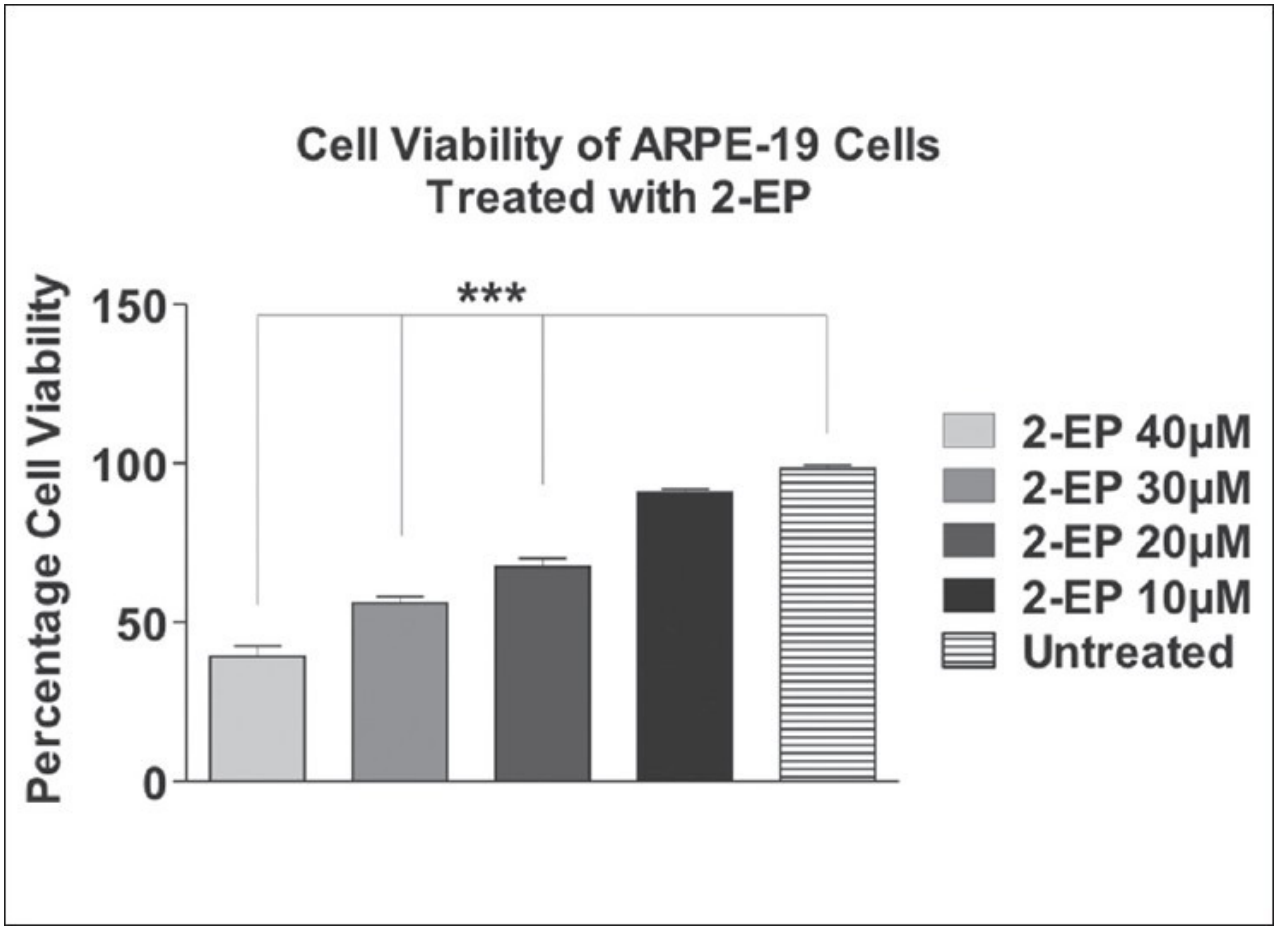
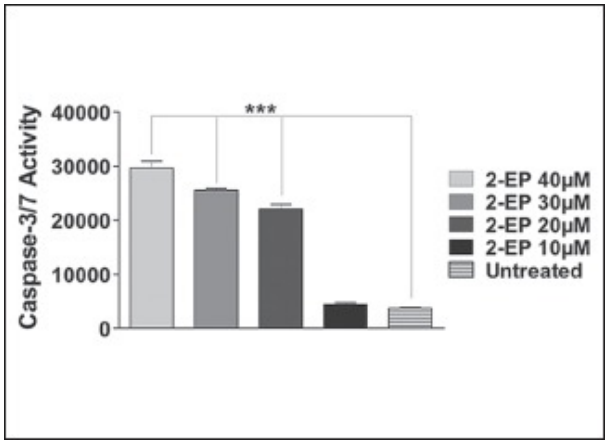


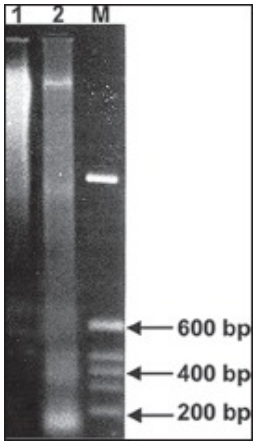
The ARPE-19 cells showed significant decrease in cell viability after 24 h treatment with 2-EP at concentrations of 40 μM, 30 μM, and 20 μM compared with untreated controls (P<0.001). The cultures treated with 10 μM of 2-EP did not show significant decrease in cell viability (P>0.05). 2-EP, 2-ethylpyridine; FNx18P<0.001.

Figure 2a



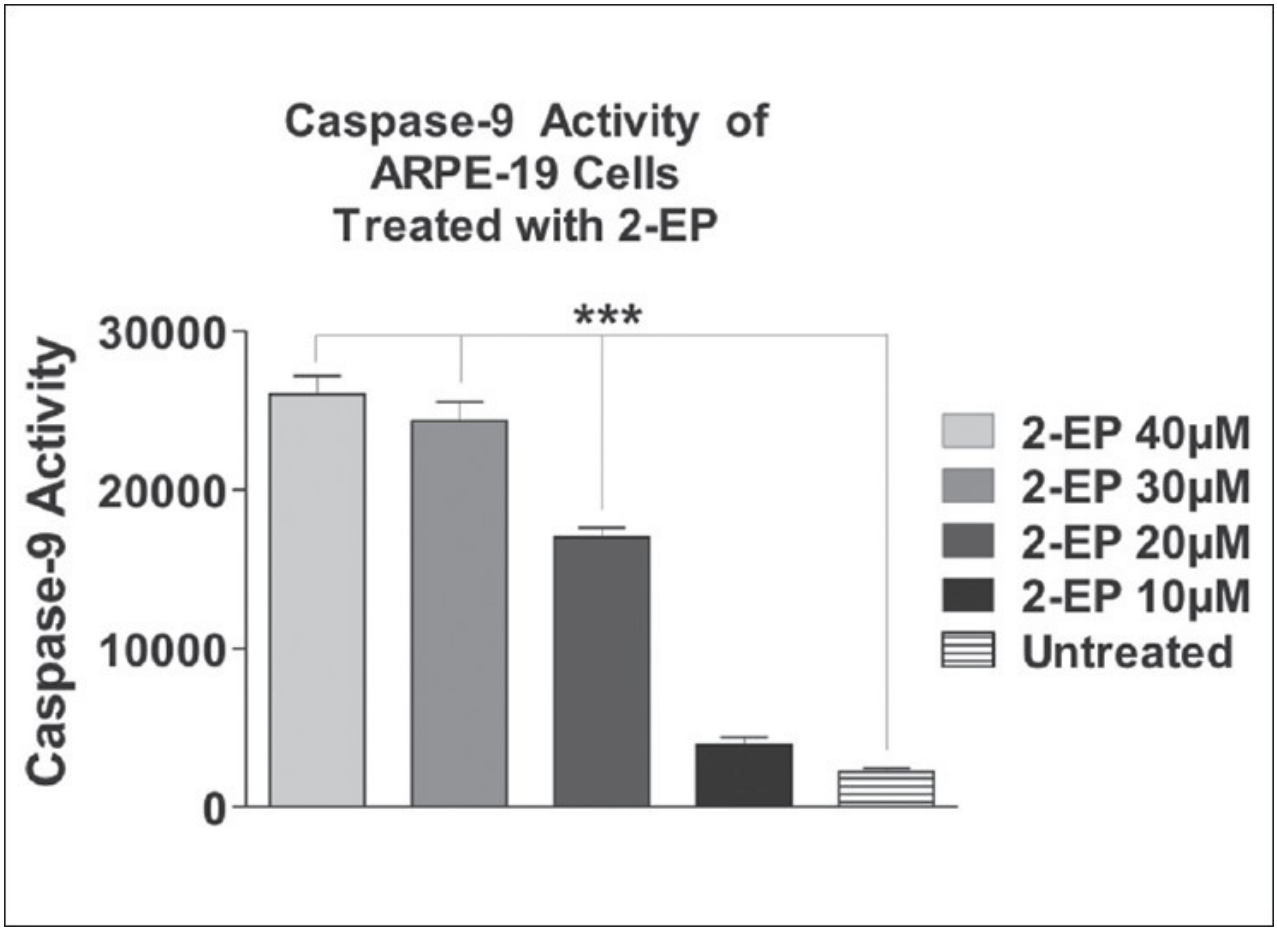
ARPE-19 cells had significantly increased caspase-3/7 activities after 24 h treatment with 2-EP at concentrations of 40 μM, 30 μM, and 20 μM, compared with untreated cultures (P<0.001). The cultures treated with 10 μM of 2-EP and untreated cultures showed minimal caspase-3/7 activities (P>0.05). 2-EP, 2-ethylpyridine; ***P<0.001.

Figure 2b



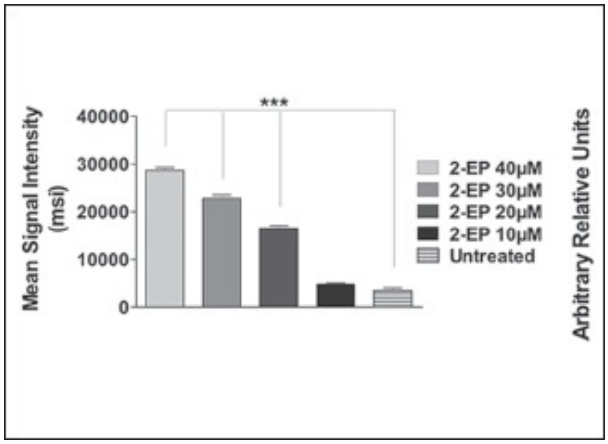
DNA fragmentation analysis showed bands at 200-bp intervals in DNA from cells treated with 2-EP at 20 μM (lane 2). The untreated DNA showed little fragmentation (lane 1). The marker showed bands at 100-bp intervals (M). 2-EP, 2-ethylpyridine. 1, untreated control sample; 2, cultures treated 24 h with 20 μM 2-EP; M, Marker

Figure 3



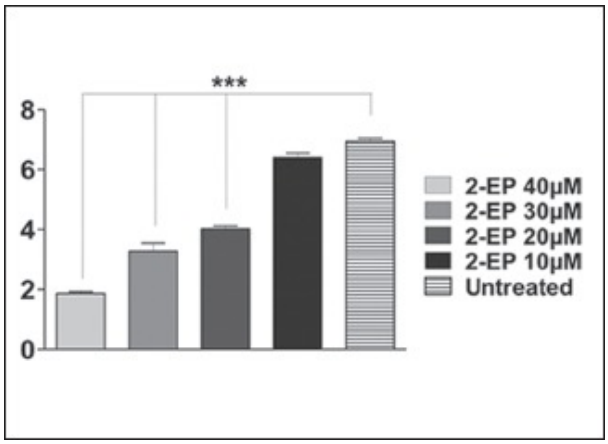
ARPE-19 cells had significantly increased caspase-9 activity after 24 h treatment with 2-EP at concentrations of 40 μM, 30 μM, and 20 μM, compared with untreated cultures (P<0.001). The cultures treated with 2-EP at 10 μM concentration and untreated culture showed minimal caspase-9 activities (P>0.05). 2-EP, 2-ethylpyridine; FNx18P<0.001

Figure 4a



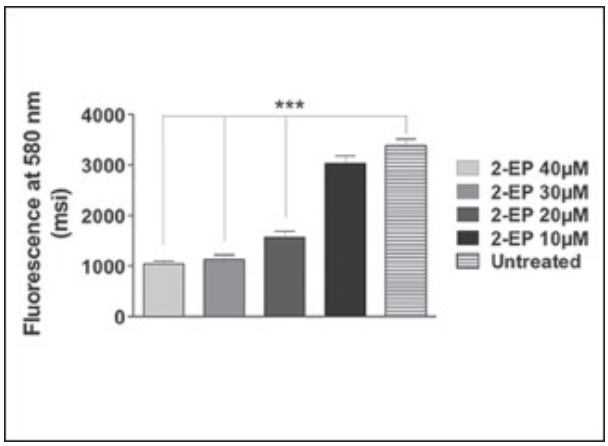
ARPE-19 cells showed significantly increased ROS/RNS levels after treatment for 24 h with 2-EP at concentrations of 40 μM, 30 μM, and 20 μM, (P<0.001) compared to the untreated cultures. The cells treated with 10ìM 2-EP and the untreated cultures showed low ROS/RNS levels (P>0.05). 2-EP, 2-ethylpyridine; ***P<0.001.

Figure 4b



ARPE-19 cells showed significantly lower red/green fluorescence representing mitochondrial membrane potential after 24 h treatment with 2-EP at concentrations of 40 μM, 30 μM, and 20 μM compared with untreated cultures (P<0.001). The cells treated with 2-EP at 10ìM concentration did not show significantly low mitochondrial membrane potentials (red/green ratio, P>0.05). 2-EP, 2-ethylpyridine. ***P<0.001.

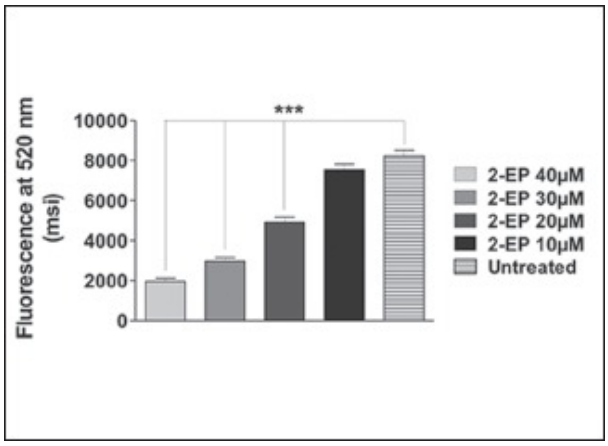
Figure 5a



ARPE-19 cells showed significantly lower redox fluorescence values after 24 h treatment with 2-EP at concentrations of

40 μM, 30 μM, and 20 μM compared with untreated cultures (*P*<0.001). The 10 μM 2-EP-treated cultures did not show a significantly lower redox fluorescence value (P>0.05). 2-EP, 2-ethylpyridine; ***P*<0.001

Figure 5b



ARPE-19 cells showed significantly lower values of the Mitotracker Green fluorescence after 24 h treatment with 2-EP at concentrations of 40 μM, 30 μM, and 20 μM compared with untreated cultures (*P*<0.001). The 10 μM 2-EP-treated cultures did show similar Mitotracker Green fluorescence values to the control cultures (P>0.05). 2-EP, 2-ethylpyridine; ***P*<0.001.