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Original Article

Protective Effects of 17β-Estradiol on Benzo(e) pyrene[B(e)P]-induced Toxicity in ARPE-19 cells

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Abstract

Purpose: The aim of this study was to examine the effect of 17β-estradiol on Benzo(e)pyrene [B(e)P]-induced toxicity in ARPE-19 cells.
Methods: We pretreated ARPE-19 cells with 20 nM and 40 nM 17β-estradiol for 6 hours, followed by addition of 300 μM B(e)*P* for additional 24 hours. Cell viability was measured using Trypan blue dye-exclusion assay. JC-1 assay was performed to measure mitochondrial membrane potential (ΔΨm). For a quantitative estimation of cell death, apoptotic markers such as caspase-3/7, caspase-9, and caspase-12 were measured.
Results: Our results demonstrated that when treated with B(e)P, the viability and ΔΨm of ARPE-19 cells declined by 25% and 63%, respectively ($P < 0.05$). However, pretreating with 17β-estradiol increased the viability of ARPE-19 cells by 21% (20 nM) and 10% (40 nM) ($P < 0.05$). Furthermore, the significantly reduced ΔΨm in βE+B(e)P treated cells ARPE-19 cells was restored by pre-treatment with 17β-estradiol- ΔΨm was increased by 177% (20 nM) and 158% (40 nM) ($P < 0.05$). We further observed a significant up-regulation in the activity of Caspases-3/7, -9, and -12 in B(e)P-treated ARPE-19 cells. However, 17β-estradiol treatment significantly reduced the activity of all apoptotic markers ($P < 0.05$).
Conclusion: In conclusion, our results demonstrate that 17β-estradiol protects ARPE-19 cells against B(e)P-induced toxicity by decreasing apoptosis, preventing cell death, and restoring mitochondrial membrane potential.

Keywords: 17β-Estradiol; Benzo(e)pyrene; Age-Related Macular Degeneration; Apoptosis; Retinal Pigment Epithelial Cells; Smoking

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INTRODUCTION

Cigarette smoking is a primary risk factor for age-related macular degeneration (AMD), which is a major cause of vision loss in the United States.[1] According to the

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Age-Related Eye Disease Study (AREDS), smoking is associated with both the dry and wet forms of AMD. Current smoking has been associated with an increased incidence of geographic atrophy and choroidal neovascularization. Furthermore, current smokers are at a higher risk of developing early AMD than both past smokers and nonsmokers.[2] A causal relation has been reported between smoking and AMD, and it has been shown that discontinuation of smoking reduces the risk for dry AMD.[3]

Cigarette smoke contains several toxic compounds including polycyclic aromatic hydrocarbons, which have been associated with the toxic effects of cigarette smoking in several diseases, including cardiovascular and respiratory diseases.[4,5] Benzo(e)pyrene [B(e)P] is a carcinogenic polycyclic aromatic hydrocarbon found in cigarette smoke.[6] A recent study reported that B(e)P induces apoptotic cell death via caspases and Bcl-family proteins in human retinal pigment epithelial cell lines.[7-9] Elevated levels of apoptosis, indicated by significantly increased number of TUNEL-positive cells, was found in AMD maculae compared to normal maculae.[10] Moreover, B(e)P is an analog of B (a) P, which has been reported to have neurotoxic effects in several *in vivo* disease models.[11-13]

Previous studies have revealed that various inhibitors such as Resveratrol, Genistein, and Memantine rescue ARPE-19 cells from apoptotic cell death.[8] Resveratrol is a plant polyphenol that increases telomere length, improves mitochondrial function, and reduces oxidative stress and vascular inflammation.[14] Genistein is a isoflavone phytoestrogen which is known to exert its neuroprotective effects by improving mitochondrial function, and by decreasing oxidative stress, inflammation, and apoptosis.[15] Memantine is a clinically relevant drug used for neurological disorders. It potentiates its actions by inhibition of NMDARs (N-methyl-D-aspartate receptors) and decreasing β-amyloid production/toxicity.[16] Furthermore, the crucial role of estrogen in protecting retinal cells against various cytotoxic compounds has been previously demonstrated.[17,18] However, to the best of our knowledge, no study has reported the protective effects of estradiol against B(e)P-induced toxicity in ARPE-19 cells. 17β-estradiol, the primary estrogen in mammals, is involved in the development of the female phenotype by regulating gene transcription and protein synthesis, and modulates the release of gonadotrophins to induce ovulation.[19] 17β-estradiol is an antioxidant hormone that is known to protect human retinal cells against cellular stress.[20]

In this study, we examined the effects of 17β-estradiol on Benzo(e)pyrene-treated ARPE-19 cells. 17β-estradiol was found to confer significant protection against the deleterious effects of Benzo(e)pyrene in the human retinal pigment epithelial cells.

METHODS

Cell Culture

The ARPE-19 cells (ATCC, Manassas, Virginia, USA) were grown in 1:1 mixture (v/v) of Dulbecco's modified Eagle's and Ham's nutrient mixture F-12; (Invitrogen-Gibco, Carlsbad, California, USA), 10 mM non-essential amino acids, 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 mg/mL, amphotericin-B 2.5 mg/mL). Our ARPE-19 cells have been validated using RPE-specific markers already. Our recent paper demonstrated significantly higher gene expression of RPE-specific markers such as bestrophin1 (BEST1), cellular retinaldehyde binding protein-1 (CRALBP), and keratin-18 (KRT18) compared to that in MIO-M1 cell lines [Supplemental Figure 1].[21]

Cells were incubated at standard conditions—37°C in 5% CO_2 and 95% relative humidity. After growing the cells to confluence, ARPE-19 cells were incubated for 24 hr in serum-free DMEM medium to make

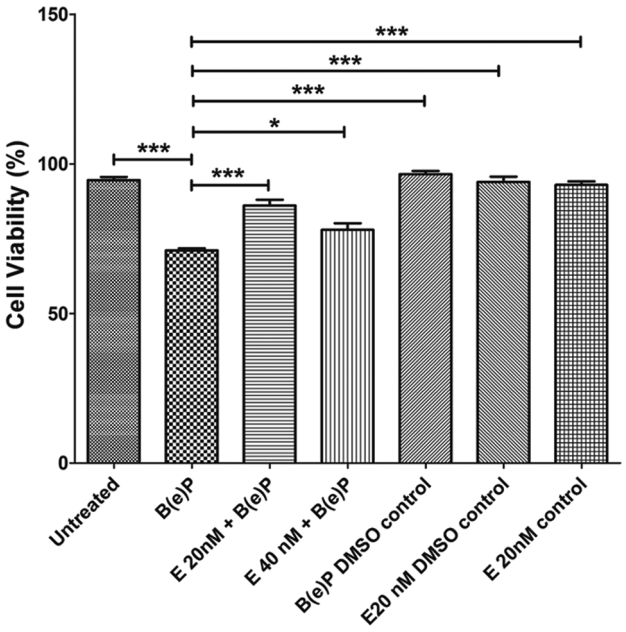

Figure 1. Effects of B(e)P and estradiol on cell viability in ARPE-19 cells: Trypan blue dye-exclusion cell viability assay showed that 300μM B(e)P decreased cell viability compared to untreated ARPE-19 cells ($P < 0.001$, $n = 4$). However, treatment with 20 nM and 40 nM estradiol (E 20nM and E 40nM respectively) increased the cell viability of B(e)P treated ARPE-19 cells ($P < 0.001$ and $P < 0.05$, $n = 4$). B(e)P equivalent DMSO control, estradiol equivalent DMSO control, and estradiol equivalent control groups showed significantly higher cell viability compared to the B(e)P-alone treated cells ($P < 0.001$, $n = 4$). All values are presented as percent (%). All data are presented as mean ± standard error of mean.

them relatively non-proliferating. Cell passage 12-15 was used; experiments were performed in triplicate, and repeated three times. "n" represents the number of times experiments were repeated in different batches of culture. Multiple wells are referred to as "replicates".

Exposure to B(e)P and 17β-Estradiol

Ten milligram of 17β-estradiol (Catalog# E8875, Sigma Aldrich, St. Louis, MO) powder was dissolved in 1mL of dimethyl sulfoxide (DMSO). Then, 100 µL of this solution was diluted to 1 ml in DMSO to get a 1 mg/ml stock solution, which was further diluted in serum free culture medium. Cells were exposed to 20 nM and 40 nM 17β-estradiol for 6 hr. B(e)P powder (Catalog# B10102, Sigma Aldrich) was dissolved in DMSO to make a stock solution which was then added to the culture media to get a 300 µM working concentration. Cells were exposed to 300 µM B(e)P+βE(β-estradiol) for 24 hr. B(e)P DMSO control and estradiol DMSO control were prepared by adding DMSO in volumes equivalent to that of B(e)P and estradiol respectively. Untreated, DMSO alone-treated, and Estradiol alone-treated cells served as controls.

Cell Viability Assay

Cells were plated, trypsinized, and harvested 24 hr after treatment with B(e)P. Cell viability was analyzed using the automated analyzer (Vi Cell; Beckman Coulter, Fullerton, CA). The analyzer performs an automated trypan blue dye-exclusion assay to measure the number of viable cells in the sample.

Mitochondrial Membrane Potential (ΔΨm) Measurements

Cells were plated at a cell density of 100,000 cells/well in 24-well plates and incubated at 37°C. Cells were exposed to B(e)P+βE for 24 hr and then incubated with the cationic JC-1 dye (5,5′,6,6′- tetrachloro1,1′,3,3′-tetraethyl-benzimidaz olylcarbocyanine iodide) (Catalog# T3168, Life Technologies, Carlsbad, CA) at 37°C for 15 min and rinsed once with 1X PBS. The fluorescent signal was measured at 525 nm (green) and 590 nm (red) wavelengths. Ratio of red to green fluorescence was calculated and analyzed.

Caspase Detection

Caspase-3/7, -9, and -12 activities were detected using FLICA apoptosis detection kit (Carboxyfluorescein FLICA Apoptosis Detection kits; Immunochemistry Technologies LLC, Bloomington, MN). The FLICA reagent has an optimal excitation range 515 to 535 nm. Apoptosis was quantified as the level of fluorescence emitted by caspase bound FLICA probes. Healthy cells appeared unstained, whereas cells undergoing apoptosis fluoresced brightly. At the designated time period, the wells were rinsed briefly with fresh culture media, replaced with 300µl/well of 1x FLICA solution in culture media, and incubated at 37°C for 1 hr. Cells were then washed with PBS. The following controls were included: untreated ARPE-19 cells without FLICA were used as a background control; untreated ARPE-19 cells with FLICA for comparison of caspase activity of treated cells; buffer alone (no cells); DMSO culture media (no cells) to exclude its cross-reaction with FLICA; and ARPE-19 cells with DMSO and FLICA to account for any cross-fluorescence between untreated cells and DMSO. Quantitative calculations of caspase activities were performed using fluorescence image scanning unit (FMBIO III; Hitachi, Yokohama, Japan). Caspase activity was measured as an average signal intensity of the fluorescence.

Statistical Analysis

One-way ANOVA followed by the post-hoc Tukey–Kramer test was used to measure differences between 3 or more experimental groups. Differences between 2 groups were analyzed using the T-test. GraphPad Prism 5.0 software (GraphPad Software, CA, USA) was used to run all statistical tests. *P* values less than 0.05 were considered statistically significant.

RESULTS

Cell Viability

Cell viability was measured using the Trypan blue dye exclusion assay. Compared to untreated cells (94.55 ± 1.07%, $n = 4$), the cell viability in 300µM B(e) P-treated ARPE-19 cells (71.05 ± 0.72%, $n = 4$) decreased by 25% ($P < 0.001$). Compared to B(e)Palone treated cells, cell viability was increased by 21% ($P < 0.001$) when pretreated with 20nM βE (86.02 ± 1.9%, $n = 4$) and by 10% ($P < 0.05$) when pretreated with 40nM βE (77.9 ± 2.2%, $n = 4$). Cell viability values in B(e)P equivalent DMSO control, estradiol equivalent DMSO control, and estradiol equivalent control were 96.5 ± 1.1% ($n = 4$), 93.9 ± 1.8% ($n = 4$), and 92.9 ± 1.19% ($n = 4$), respectively [Figure 1].

Mitochondrial Membrane Potential (ΔΨm)

Mitochondrial Membrane Potential (ΔΨm) was measured using JC-1 assay. Compared to untreated cells (5.94 ± 0.57 arbitrary units (a.u.)), ΔΨm of 300 µM B(e)P treated cells (2.18 ± 0.17 a.u., $n = 3$) was reduced by 63% ($P < 0.001$). Compared to B(e)P-alone treated cells, ΔΨm was significantly higher when cells were pretreated with 20 nM βE + B(e)P (177%, 6.04 ± 0.07 a.u.; $P < 0.001$, $n = 6$) and 40nM βE + B(e)P (158%, 5.63 ± 0.38 a.u.; $P < 0.001$, $n = 6$). B(e)P equivalent

DMSO control had ΔΨm of 6.06 ± 0.40 a.u. (n = 6), estradiol equivalent DMSO control had ΔΨm of 4.4 ± 0.45 a.u. (n = 4), estradiol equivalent control had ΔΨm of 3.4 ± 0.36 a.u. (n = 4), and untreated control cultures had ΔΨm of 5.94 ± 0.58 a.u. (n = 6) [Figure 2].

Caspase- 3/7 Activity

Compared to untreated cells (1512.6 ± 120.7 a.u., n = 3), treatment with 300 μM B(e)P (17258.96 ± 552.5 a.u., n = 3) significantly increased the activity of caspase-3/7 by 1041% (P < 0.001). However, caspase-3/7 levels decreased when exposed to 20 nM βE + B(e)P (63%, 6385.6 ± 132.3 a.u.; n = 3) (P < 0.001) and 40 nM βE + B(e)P (30%, 12060.8 ± 611.2 a.u., n = 3) (P < 0.01) compared to B(e)P alone. Caspase 3/7 levels in B(e)P equivalent DMSO control, estradiol equivalent DMSO control, and estradiol equivalent control were 2817.2 ± 395.8 a.u. (n = 3), 3424 ± 598.6 a.u. (n = 3), and 3496.4 ± 473.2 a.u. (n = 3), respectively [Figure 3].

Caspase-9 Activity

Compared to untreated cells (2372.7 ± 435.17 a.u., n = 4), treatment with 300 μM B(e)P (26556.04 ± 601.48 a.u., n = 3) significantly increased the activity of caspase-9 by 1019% (P < 0.001). Compared to B(e)P-alone treated cells, caspase-9 levels decreased when cells were exposed to βE i.e. 20nM βE + B(e)P (23%, 20409.7 ± 2820.9 a.u., P = 0.05, n = 4) and 40 nM βE + B(e)P (30%, 18609.7 ± 915.6 a.u., P < 0.01, n = 4). Caspase-9 levels in B(e)P equivalent DMSO control, estradiol equivalent DMSO control, and estradiol equivalent control were 2094.6 ± 812.7 a.u. (n = 4), 1152.5 ± 536.7 a.u. (n = 4), and 2590 ± 240.7 a.u. (n = 3), respectively [Figure 4].

Caspase-12 Activity

Treatment with 300 μM B(e)P caused a significant increase in caspase-12 activity (20815.6 ± 880.09 a.u., P < 0.001, n = 3) compared to untreated cells (88.8 ± 526.7 a.u.). Caspase-12 levels were reduced when exposed to estradiol: 20 nM βE + B(e)P (16%, 17476.8 ± 409.4 a.u., n = 3) (P < 0.05), and 40 nM βE + B(e)P (27%, 15141.4 ± 234.3 a.u., n = 3) (P < 0.01). Caspase-12 levels in B(e)P equivalent DMSO control, estradiol equivalent DMSO control, and estradiol

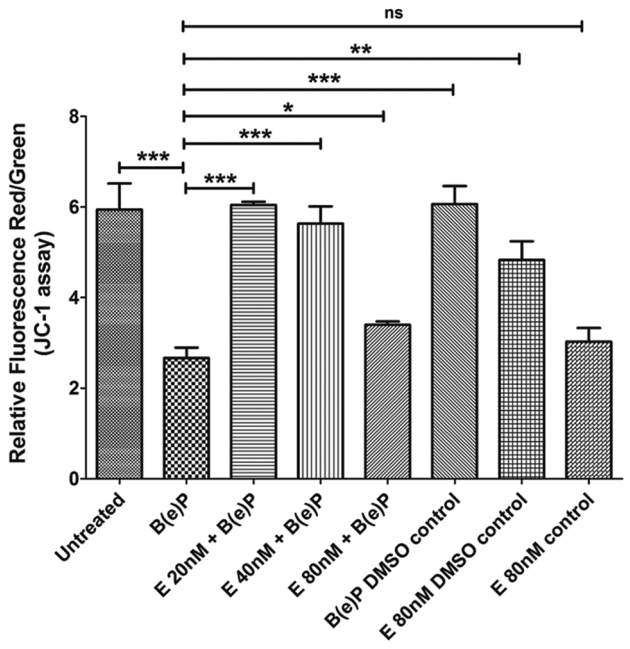

Figure 2. Effects of B(e)P and estradiol on mitochondrial membrane potential in ARPE-19 cells: JC-1 assay results demonstrated that compared to untreated ARPE-19 cells, the mitochondrial membrane potential (ΔΨm) was reduced with 300μM B(e)P alone-treated cells (P < 0.001, n = 6). Addition of 20nM and 40nM estradiol significantly increased the ΔΨm in B(e)P ARPE-19 cells (P < 0.05, n = 6). Compared to B(e)P alone-treated cells, elevated mitochondrial membrane potential was found in B(e)P equivalent DMSO control, estradiol equivalent DMSO control, and estradiol equivalent control groups (P < 0.05, n = 6). All values are presented in arbitrary units (a.u.). All data are presented as mean ± standard error of mean.

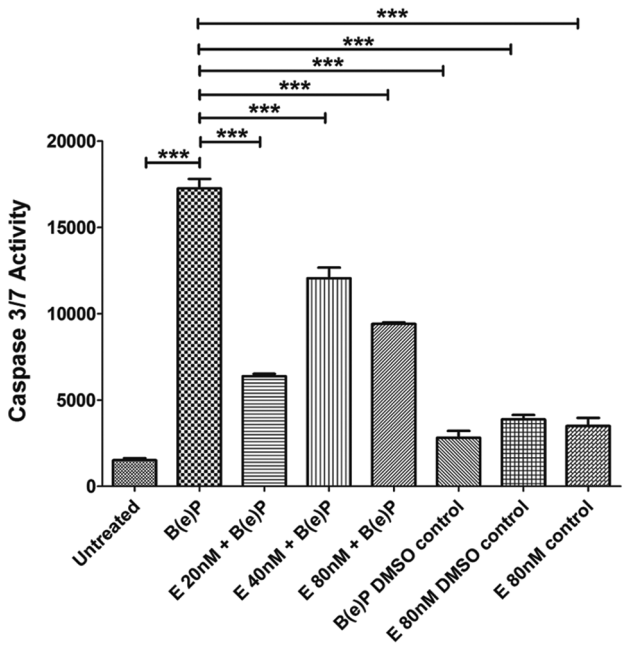

Figure 3. Effects of B(e)P and estradiol on caspase-3/7 activity in ARPE-19 cells: The FLICA caspase-3/7 assay results demonstrated significantly elevated caspase-3/7 activity in B(e)P-alone treated ARPE-19 cells (P < 0.001, n = 3) compared to untreated cells. Pretreatment with 20 nM and 40 nM estradiol led to drastic decrease in caspase-3/7 activity in B(e)P-treated cells (P < 0.001, n = 3). In addition, caspase-3/7 activity was reduced significantly in estradiol equivalent control, estradiol equivalent DMSO control, and in B(e)P equivalent DMSO control groups (P < 0.001, n = 3). All values are presented in arbitrary units (a.u.). All data are presented as mean ± standard error of mean.

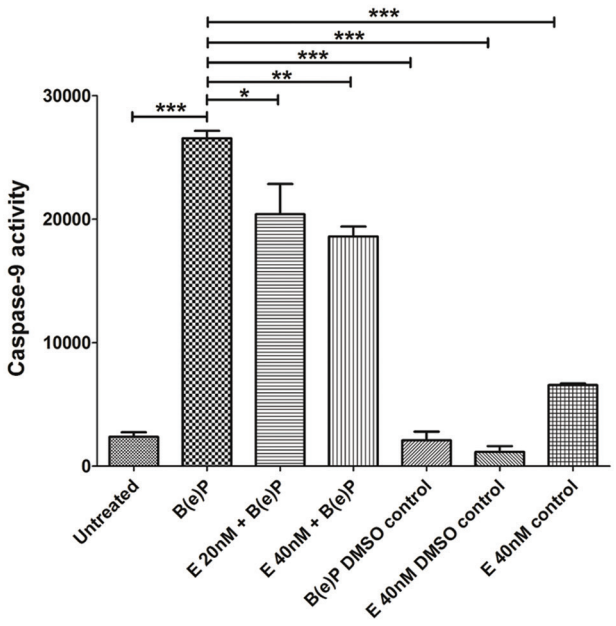

Figure 4. Effects of B(e)P and estradiol on caspase-9 activity in ARPE-19 cells: B(e)P-alone treated ARPE-19 cells had up-regulated caspase-9 activity levels compared to the untreated cells ($P < 0.001$, $n = 3$–4). Treatment with 20 nM and 40 nM estradiol decreased caspase-9 activity in B(e)P ARPE-19 cells ($P < 0.05$, $n = 4$). Furthermore, caspase-9 activity was decreased significantly in estradiol equivalent control, estradiol equivalent DMSO control, and in B(e)P equivalent DMSO control groups ($P < 0.001$, $n = 3$). All values are presented in arbitrary units (a.u.). All data are presented as mean ± standard error of mean.

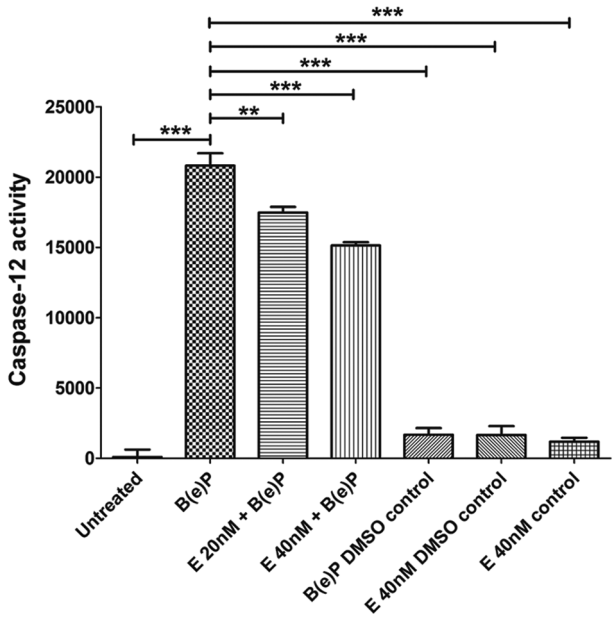

Figure 5. Effects of B(e)P and estradiol on caspase-12 activity in ARPE-19 cells: Compared to the untreated cells, treatment with B(e)P-alone led to increased caspase-12 activity in ARPE-19 cells ($P < 0.001$, $n = 3$). Treatment with 20 nM and 40 nM estradiol reduced caspase-12 activity in B(e)P ARPE-19 cells ($P < 0.05$, $n = 3$). Significant reduction in caspase-12 activity was observed in estradiol equivalent control, estradiol equivalent DMSO control, and in B(e)P equivalent DMSO groups ($P < 0.001$, $n = 3$). All values are presented in arbitrary units (a.u.). All data are presented as mean ± standard error of mean.

equivalent control were 1676.5 ± 471.8 a.u. ($n = 3$), 1656.9 ± 627.9 a.u. ($n = 3$), and 1189.9 ± 266.9 a.u. ($n = 3$), respectively [Figure 5].

DISCUSSION

The present study demonstrated the protective role of 17β-estradiol in rescuing ARPE-19 cells from B(e) P -induced toxicity. Pretreatment with 17β-estradiol prevented cell loss and collapse of mitochondrial membrane potential in B(e)P –pretreated ARPE-19 cells. Furthermore, reduced apoptosis caused by 17β-estradiol was evident, since there was a decline in the activity of caspases-3/7, -9, and -12 in B(e)P -treated ARPE-19 cells.

Similar to the results of our study, previous studies have demonstrated that 17β-estradiol protects ARPE-19 cells against hydrogen peroxide-induced oxidative stress.[22] 17β-estradiol is neuroprotective and prevents loss of retinal ganglion cells in a glaucoma model.[23] It has been reported that the antioxidant activity of 17β-estradiol protects neurons from oxidative stress-induced cell death *in vitro* in a mouse hippocampal cell line - HT22.[24] In that study, 17β-estradiol protected neurons by blocking glutamate-mediated DNA degradation. Moreover, administration of 17β-estradiol protects against retinal ischemia-induced tissue damage in rats.[25] In general, the role of estrogen as a neuroprotective agent has been well-established.[26] Recent studies have highlighted the neuroprotective effect of estrogens on retinal neurons *in vitro* against oxidative stress.[27] Estrogen has been reported to protect against cataract development and uveitis.[28]

Previous studies have shown that younger women have higher levels of estrogen compared to the aging and post-menopausal women.[29] Furthermore, smoking is known to diminish the protective ability of estrogen.[30] Therefore, it could be speculated that aging-related decline in estrogen levels might be increasing the susceptibility to the toxic effects of cigarette smoking. Hormone replacement therapy has been reported to lower intraocular pressure in menopausal women,[31] and improve cognitive function.[32] Furthermore, postmenopausal hormone therapy is known to increase retinal blood flow and protects the retinal nerve fiber layer.[30] Evidently, estrogen has been implicated to be beneficial in the treatment of various visual disorders.

B(e)P is known to cause toxicity by mediating apoptotic cell death and DNA fragmentation in ARPE-19 cells.[33] It has been reported that 17β-estradiol,

an antioxidant, mediates its cytoprotective effects via preservation of mitochondrial function and reduction of apoptosis in ARPE-19 cells.[7] Consistent with those findings, our study revealed that 17β-estradiol prevented cell death and preserved mitochondrial function by restoring mitochondrial membrane potential in B(e)P treated ARPE-19 cells. In addition, our results demonstrated that 17β-estradiol led to a substantial decrease in the activity of caspases in B(e)P treated ARPE-19 cells. Caspases are normally present in cells as inactive proenzymes, and activation of caspases is a biochemical hallmark of apoptosis.[34] In order to quantitate the additive effects of 17β-estradiol and B(e)P on apoptotic activity, we used the FLICA probes which are cell permeant, non-cytotoxic fluorescent labeled inhibitors of caspases. These probes covalently and irreversibly bind to active caspase enzymes. Apoptotic cells which retain a higher concentration of FLICA, exhibit brighter green fluorescence compared to healthy cells. In our study, treatment with 17β-estradiol led to a drastic decline in the activity caspase-9, an initiator caspase, in B(e)P treated ARPE-19 cells. Cellular stress causes release of mitochondrial cytochrome c into the cytosol. Cytochrome c binds APAF-1, an adaptor protein, which in turn recruits caspase-9 to form a multiprotein complex called the apoptosome. Once activated, caspase-9 is cleaved and activates executioner/effector caspases, which eventually leads to the degradation of cellular components.[35] The current study also demonstrated that in B(e)P treated ARPE-19 cells, 17β-estradiol lowered the activity of caspase-3/7, and caspase-12. Caspase-3 and caspase-7 are effector caspases that are closely related to each other and are universally activated in response to cell death stimuli in various cell types including retinal cells. Caspase-3 is activated in the apoptotic cell both by extrinsic (via death ligand), and intrinsic (mitochondrial) pathways, and is known to mediate feedback processing of caspase-9.[36] Caspase-12 is known to be activated by ER stress and has been linked to inflammatory responses.[37,38] Our results are consistent with previous studies by Lee et al and Pelzer et al that reported that 17β-estradiol caused a decline in apoptosis via reduction of caspase activity in hepatocytes and cardiac myocytes respectively.[39,40]

To summarize, this study presents important findings that emphasize the prominent role of 17β-estradiol in protecting human retinal pigment epithelial cells against the toxic effects of Benzo(e)pyrene. These results suggest that administration of 17β-estradiol may be considered to prevent retinal cell damage caused by cigarette smoke. Further studies are required to examine the mechanisms by which 17β-estradiol mediates its protective effects against B [e] p-induced cellular toxicity in ARPE-19 cells.

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Conflicts of Interest

There are no conflicts of interest.

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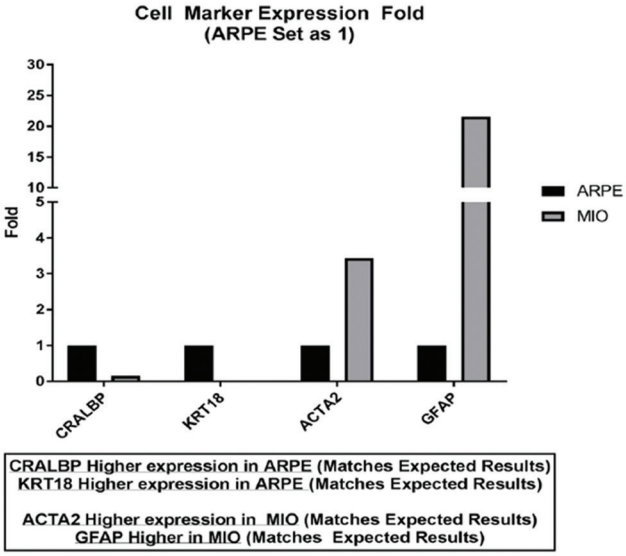
Gene Symbol	Gene ID	RefSeq ID	Exon Coverage	
RLBP1 (CRALBP)	6017	NM_000326	2-3	ARPE Marker
KRT18	3875	NM_000224	3-4	ARPE Marker
ACTA2	59	NM_001141945	8-9	MIO Marker
GFAP	2670	NM_002055	6-8	MIO Marker

All values normalized to ARPE19

	RLBP1 (ARPE Marker)	KRT18 (ARPE Marker)	ACTA2 (MIO Marker)	GFAP (MIO Marker)
ARPE19	1	1	1	1
MIO	0.1563	0.016135	3.44	21.6
	ARPE 6.39 fold higher	ARPE 62.0 fold higher	MIO 3.44 fold higher	MIO 21.6 fold higher



Supplemental Figure 1. ARPE-19 CellMarkers.