Cytotoxicity of Ofloxacin To Human Corneal Epithelial Cells By Inducing Cell Cycle Arrest And Mitochondrion Dependent Apoptosis

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Abstract

Ofloxacin (OFX), a second-generation fluoroquinolone, has been widely used as a synthetic antibiotic in eye clinic. However, its effect on human corneal epithelium (HCEP) and the probable underlying mechanisms are unclear. This study was conducted to define the cytotoxicity of OFX and the underlying mechanisms using an in vitro model of HCEP cells. After HCEP cells were treated with different concentrations of OFX, the cytopathic effect (CPE) and viability were assessed by light microscopy and MTT assay, cell cycle arrest and apoptosis were detected by Flow cytometry, AO/EB double-staining, gel electrophoresis and transmission electron microscopy, and caspase activation, mitochondrial transmembrane potential (MTP) disruption and apoptotic protein expression were quantified using ELISA, FCM and Western blot. Our results showed that OFX above concentrations of 0.0375% induced CPE formation and viability decline of HCEP cells in a dose- and time-dependent manner. OFX arrested the cells at S phase and induced plasma membrane permeability elevation, phosphatidylserine externalization, DNA fragmentation, and apoptotic body formation. Furthermore, activations of caspase-2, -9, and -3, up-regulation of Bax, Bad, cytochrome c and apoptosis inducing factor (AIF), down-regulation of Bcl-2 and Bcl-xl, and disruption of MTP were also found in OFX induced HCEP cell apoptosis. In conclusion, OFX above 1/8 of its therapeutic concentration has inconsiderable cytotoxicity to HCEP cells by inducing cell cycle arrest and apoptosis which is regulated by a death receptor-mediated mitochondrion-dependent signaling pathway.

Keywords: Ofloxacin; Human corneal epithelial cells; Cytotoxicity; Apoptosis; Mitochondron

Abbreviations: AIF: apoptosis inducing factor; AO: acridine orange; CPE: cytopathic effect; EB: ethidium bromide; FCM: Flow cytometry; HCEP: human corneal epithelium; JC-1: 5,5',6,6'-Tetrachloro-1,1',3,3'-Tetraethylbenzimid; MTP: mitochondrial transmembrane potential; MTT: thiazolyl blue tetrazolium bromide; OFX: ofloxacin; PI: propidium iodide; PS: phosphatidylserine; TEM: Transmission Electron Microscopy

Introduction

The cornea, an vascular transparent barrier in the outmost of the eye, plays critical roles in maintaining our vision, and any damages to it may lead to corneal opacification, visual impairment, and even blindness [1]. Among the five layer structures of cornea, the epithelium is composed of multilayer epithelial cells, including three or four layers of squamous cells with flattened nuclei, two or three layers of polyhedral prickle cells, and one layer of columnar basal cells [2]. Under normal condition, superficial squamous cells age and slough off into the tear, and inferior basal and wing cells divide and migrate to the anterior of the cornea to replace the lost cells regularly. This homeostasis of the epithelial cells is maintained by the proliferation and differentiation of limbal epithelial stem cells [3]. Physiologically, the outmost epithelium forms a physical barrier to prevent noxious agents from infecting and damaging the eye [4]. Since the epithelium

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is more and more in direct contact with drugs, pathogens and biomaterials, disruption of its barrier function can cause ocular irritation and may be a risk factor for microbial infections and drug damages [5-7].

Ofloxacin (OFX), a second-generation fluoroquinolone with a broad spectrum of activity against gram-positive and gram-negative bacteria by inhibiting topoisomerase II and IV, is a potent topical agent against various ocular bacterial species and extensively used in eye clinic for antibacterial treatment [8-11]. OFX has been reported to have toxic effect on human corneal epithelium (HCEP) in vivo, and have inhibiting effects on the proliferation and viability of HCEP cells in vitro [12,13]. However, the possible cellular and molecular mechanisms of the cytotoxicity of OFX to HCEP cells have not yet been clarified. One of the obstacles in studying intensively the cytotoxicity of OFX is the lack of an in vitro model that can be used to investigate the possible cytotoxic mechanisms and the prospective therapeutic interventions [14,15].

As we know, cytotoxicity study using an in vitro model of cultured HCEP cells is indispensable to evaluate the unwanted effects of drugs before drawing any final conclusions from clinical and human utilization [16]. As cultured HCEP cells could provide an effective model for in vitro cytotoxicity studies, the recently established non-transfected HCEP cell line make it possible to study the cytotoxicity of OFX and its possible cellular and molecular mechanisms as well [17]. The present study was intended to investigate the cytotoxicity of OFX and its underlying cytotoxic mechanisms using an in vitro model of non-transfected HCEP cells.

Materials and Methods

Chemicals: The Dulbecco’s modified Eagle medium: Ham’s nutrient mixture F-12 medium (DMEM/F12, 1:1), fetal bovine serum (FBS) and trypsin for cell culture were from Invitrogen (Carlsbad, CA, USA). FITC-labeled Annexin V Apoptosis Detection Kit I and propidium iodide (PI) were from BD Biosciences (San Jose, CA, USA). Quick Tissue/Culture Cells Genomic DNA Extraction Kit was from Dongsheng Biotech (Guangzhou, China). Rabbit anti-human caspase-2, -3, and -9 (active form) antibodies were from Biosynthesis biotechnology (Beijing, China). HRP-labeled goat anti-rabbit secondary antibody was from ComWin Biotech Co., Ltd. (Beijing, China). Ofloxacin (OFX, C_{18}H_{20}FN_{3}O_{4}) and all other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

In vitro culture and treatment: HCEP cells, from a non-transfected HCEP cell line (uHCEPC01) established previously in our laboratory [17], were maintained and cultured in 10% heat-inactivated FBS-containing DMEM/F12 medium at 37°C in 25 cm² culture flasks. For treatment, the culture medium of HCEP cells at logarithmic phase was replaced entirely with the medium containing OFX at various concentrations. HCEP cells cultured in the same medium without any OFX addition at the same time point were used as controls in all experiments.

Light microscopic observations: HCEP cells were inoculated into a 24-well culture plate and cultured in 10% BCS-DMEM/F12 medium at 37°C in a humidified 5% CO₂ incubator. Logarithmic HCEP cells were treated with OFX at concentrations from 0.01875% to 0.3% as described above. The cell morphology and cytopathic effect (CPE) were monitored with an inverted light microscope (model TS100, Nikon, Tokyo, Japan) every 4h.

Cell viability assay: Cell viability was measured by thiazolyl blue tetrazolium bromide (MTT) according to the method described previously [18]. Briefly, HCEP cells were seeded into a 96-well culture plate at a density of 1×10⁴ cells/well, and were treated as described above. The culture medium was replaced, every 4h, by 200 μl serum-free DMEM/F12 medium containing 1.1 mM MTT, and then incubated 4h at 37°C in dark. After 150 μl of dimethyl sulfoxide (DMSO) was added, the 490 nm absorbance of each well was measured with a microplate reader (model Multitcan GO, Thermo Scientific, MA, USA).

Plasma membrane permeability assay: Plasma membrane permeability was measured by acridine orange (AO)/ethidium bromide (EB) double-staining method as described previously [19]. Briefly, HCEP cells were cultured and treated as previously above. Then the cells were harvested and stained with 100 μg/ml AO/EB (1:1) solution for 1 min, and observed under a fluorescent microscope (model...
The cells with red or orange nuclei were designated as apoptotic cells while those with green nuclei as non-apoptotic cells, and their apoptotic ratio was calculated according to the formula: "Apoptotic rate (%) = Apoptotic cells / (Apoptotic cells + non-apoptotic cells) x 100" with at least 400 cells counted in each group.

**DNA fragmentation assay:** DNA fragmentation was examined by agarose gel electrophoresis as previously described [19]. In brief, HCEP cells in 25 cm² flasks were cultured and treated as described above. After the cells were harvested and washed with ice-cold phosphate-buffered saline (PBS), their DNA was isolated with a Quick Tissue/Culture Cells Genomic DNA Extraction Kit (CWbiotech, Beijing, China). The DNA preparation from each group was electrophoresed on a 1% agarose gel (200 mA, 260 min), stained with 0.005% ethidium bromide for 10 min, and observed with an imaging system (model EC3, UVP, LLC Upland, CA, USA).

**Transmission Electron Microscopy (TEM):** The ultra structure of HCEP cells was characterized by TEM following the method reported previously [19]. Briefly, HCEP cells in 25 cm² flasks were cultured, treated with 0.1% OFX and harvested as described above. After fixed successively with 4% glutaraldehyde and 1% osmium tetroxide, the cells were dehydrated and embedded in epoxy resin. Ultrathin sections were stained with 2% uranyl acetate-lead citrate and observed by a transmission electron microscope (model H700, Hitachi, Tokyo, Japan).

**Flow cytometry (FCM) analyses:** The cell cycle progression, phosphatidylserine (PS) externalization and mitochondrial transmembrane potential (MTP) were detected and analyzed by FCM as reported previously [18]. In brief, HCEP cells in 6-well plates were cultured, treated and harvested as described above. After washed twice with 1 ml PBS by centrifugation (200g, 10 min), the cells were fixed with 70% alcohol overnight at 4°C. After that, the cell suspension was stained with PI for cell cycle assay, stained with Annexin-V/PI using FITC Annexin V Apoptosis Detection Kit I for PS externalization assay, and stained with 10 µg/ml JC-1 (5,5’,6,6’-Tetrachloro-1’,3’,3’-Tetraethylbenzimida) for MTP assay, respectively. The stained HCEP cells were detected by a flow cytometer (model FAC Scan, BD Biosciences, San Jose, CA, USA).

**Caspase activation assay by ELISA:** Caspase activation was detected by ELISA as described previously [18]. Briefly, HCEP cells in 25 cm² flasks were cultured treated with 0.1% OFX and harvested every 2h as described above. Whole-cell protein extracts were prepared and coated into the wells of a 96-well microtiter plate. Following three-washes with PBS containing 0.05% Tween-20 (PBST), the wells were blocked with 5% non-fat milk, incubated with rabbit anti-human caspase-2, -3, and -9 (active form) antibodies (1:500), and the HRP-conjugated goat anti-rabbit secondary antibody (1:3000), respectively at 37°C for 2h. Following three washes, a colorimetric reaction was induced by 1% tetramethylbenzidine substrate for 25 min at room temperature in the dark. Color development was stopped with 50 µl 0.5 M H₂SO₄, and the 490 nm absorbance of each well was measured using a Multiskan 60 microplate reader (Thermo Scientific).

**Western blot:** Western blot was used to quantify the expression of apoptosis related proteins. HCEP cells in 25 cm² flasks were treated 8h with 0.3% OFX and harvested as described above. Cytoplasmic extracts were prepared using mitochondria and cytoplasmic protein extraction kit (Sangon biological engineering, Shanghai, China). The protein extract (about 45 µg) from the same number of cells in each group was electrophoresed by 10% SDS-PAGE, and transferred to PVDF membranes. The membrane was blocked overnight at 4°C using 5% nonfat milk in 0.05% Tween-20 containing Tris buffer (10 mM Tris, 150 mM PBS, pH 8.0). Following three washes with the same buffer, the membrane was incubated with rabbit anti-human IgG monoclonal antibody to cytochrome c, and apoptosis-inducing factor (AIF), Bax, Bad, Bcl-2, Bcl-xL and β-actin (all in 1:1000 dilution) at 37°C for 60 min, respectively. Following three washes, the membrane was incubated with 100 µl of HRP-conjugated goat anti-rabbit IgG monoclonal antibody (1:3000) at 37°C for 90 min. Following three washes, the membrane was incubated with DAB solution (3 mg DAB, 10 µl of 30% H₂O₂, 4.5 ml of 10 mM Tris buffer) for 25 min at room temperature in the dark, and observed with an EC3 imaging system (UVP). The optical density of each band was quantified using image analysis software (Image J v1.4.8, NIH, MD, USA).

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Statistical analysis: Each experiment was repeated 3 times independently. Data were presented as mean ± SEM and analyzed for statistical significance with one way analysis of variance (ANOVA). Differences to controls were considered statistically significant when $P < 0.05$.

Results and Discussion

Cytotoxic effect of OFX on HCEP cells: To evaluate the cytotoxicity of OFX, the morphology and cell viability of HCEP cells were first examined by light microscopy and MTT assay. Morphological observations showed that HCEP cells treated with 0.0375%-0.3% of OFX exhibited typical CPE in a dose- and time-dependent manner, while no obvious difference was observed between those treated with OFX below the concentration of 0.01875% and controls (Figure 1A). Our results MTT assay revealed that the cell viability of HCEP cells decreased with time and concentration after exposed to OFX above the concentration of 0.0375% ($P < 0.01$ or $0.05$), while that of HCEP cells treated with OFX below the concentration of 0.01875% showed no significant difference to controls (Figure 1B). All these demonstrate that OFX at concentrations above 0.0375% (1/8 of its therapeutic concentration) has a dose- and time-dependent cytotoxicity by inducing CPE formation and viability decline of HCEP cells, which is supported by previous reports on the inhibiting effects of OFX on the proliferation and viability of HCEP cells in vitro [13].

**Figure 1:** OFX induces cytopathic effect and viability decline of human corneal epithelial cells.

A: Light microscopy. One representative photograph from three independent experiments is shown, and the dosage and exposure time of OFX are shown in the top-left of each photograph. Scale bar, 100 μm.

B: MTT assay. The cells were treated with the indicated concentrations and exposure time of OFX. Values of cell viability are presented as Mean ± SEM (n=3). *, $P < 0.05$; **, $P < 0.01$ vs control untreated cells.

OFX induces cell cycle arrest of HCEP cells: To postulate the cytotoxic mechanisms of OFX, we then detected the cell cycle progression of HCEP cells by FCM using PI staining. Our results showed that the number of HCEP cells at G1 phase decreased with time ($P < 0.01$), that at S phase increased with time ($P < 0.05$ or 0.01), and that at G2/M phase decreased with time ($P < 0.05$ or 0.01) after treated with 0.3% OFX for 8h, 12h, and 16h, respectively (Table 1). These indicate that OFX could induce S phase arrest of HCEP cells in cell cycle progression. Since cell viability decline and cell cycle arrest are often related with apoptosis triggered by chemotherapeutic agents [20-24], it can be hypothesized that the cytotoxicity of OFX to HCEP cells might be related with apoptosis.

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Table 1: Effects of ofloxacin on cell cycle parameters of HCEP Cells.
Data was expressed as means ± SEM (n = 3). *P < 0.05, **P < 0.01 vs control untreated cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of cells (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>None (control)</td>
<td>72.92 ± 2.47</td>
</tr>
<tr>
<td>Ofloxacin (0.3%)</td>
<td></td>
</tr>
<tr>
<td>8h</td>
<td>74.90 ± 3.15</td>
</tr>
<tr>
<td>12h</td>
<td>62.94 ± 3.24**</td>
</tr>
<tr>
<td>16h</td>
<td>60.58 ± 3.99**</td>
</tr>
</tbody>
</table>

OFX induces apoptosis in HCEP cells: As well demonstrated, plasma membrane permeability elevation, PS externalization, DNA fragmentation and apoptotic body formation are the hallmark features of apoptosis [25-28]. To verify whether the cytotoxicity of OFX was achieved by inducing cell apoptosis, we also examined the plasma membrane permeability, PS externalization, DNA fragmentation and apoptotic body formation of HCEP cells using AO/EB double staining, FCM, gel electrophoresis and TEM, respectively. Our AO/EB double staining showed that OFX above the concentration of 0.0375% could elevate the membrane permeability of HCEP cells with concentration and time (P < 0.01 or 0.05), while that of HCEP cells exposed to OFX below the concentration of 0.01875% showed no significant difference to controls (Figure 2A). FCM using FITC-Annexin-V/PI staining displayed that the number of apoptotic cells increased to 18.10 ± 0.61% (P < 0.01), 61.32 ± 0.97% (P < 0.01), and 99.04 ± 0.77% (P < 0.01) after treated with 0.3% OFX for 8h, 12h, and 16h, respectively, when compared with its corresponding control (Figure 2B). DNA electrophoresis indicated that typical DNA ladders were found in the HCEP cells treated with 0.075%-0.3% OFX, while no DNA ladder was found in those of controls (Figure 2C). Under TEM, ultrastructural disorganization were also found in the cells treated with 0.3% OFX, including microvillus lost, cytoplasmic vacuolation, mitochondrion swelling, chromatin condensation, and apoptotic body formation (Figure 2D). To our knowledge, the formation of apoptotic bodies with PS externalization in plasma membrane is a vital feature for macrophages to clear away the apoptotic cells by phagocytosis, which is the key difference between apoptosis and necrosis [25,28]. Therefore, all these results confirmed that OFX could induce apoptosis of HCEP cells by plasma membrane permeability elevation, PS externalization, DNA fragmentation and apoptotic body formation. The OFX induced apoptosis is consistent with our previous reports of pranoprofen and betaxolol induced apoptosis in human corneal endothelial cells [19,29], and the other researchers’ reports of OFX in rabbit and canine chondrocytes [22,24].

OFX induces caspase activation in HCEP cells: Generally, apoptosis is a normal physiological process regulated orderly by intricate pathways and is triggered either by an extrinsic pathway via the ligation of death receptors or an intrinsic pathway emerges from mitochondria [30-32], both of which result in the activation of initiator caspases (e.g., caspase 2, 8, 10, and 9) required for executioner caspase (e.g., caspase 3, 6, and 7) activation [33,34]. To postulate the possible signaling pathways involved in OFX-induced apoptosis, we detected the activation of caspase-2, -3, and -9 using their active forms of antibodies in the present study. Our results of ELISA using the active forms of caspase-2, -3, and -9 antibodies indicated that caspase-2, -3, and -9 in 0.3% OFX treated HCEP cells were successively activated (P < 0.01 or 0.05) (Figure 3).

As we show, caspase-2 was first activated to its peak value at 8 h (P < 0.01), while caspase-9 and -3 activated to their peak value at 12 h (P < 0.01). These display that OFX could induce activation of caspase-2, -3, and -9 in HCEP cells. Since caspase-2 is an important regulator of extrinsic apoptotic pathway related to TNFR1 while caspase-9 is one of the key regulators of the mitochondrion-dependent intrinsic apoptotic pathway [33], the activation pattern of the above caspases suggest that the apoptosis of HCEP cells induced by OFX might be mediated by both a death receptor-mediated pathway and a mitochondrion-dependent pathway. The involvement of both an extrinsic death receptor-mediated signaling pathway and an intrinsic mitochondrion-dependent signaling pathways has also been reported in chemical-induced apoptosis [18,22,24,35].

**Figure 2:** OFX induces apoptosis of human corneal epithelial cells.

A: Acridine orange/ethidium bromide (AO/EB) double staining. The cells were treated with the indicated concentrations and exposure time of OFX. Values of apoptotic ratio are expressed as mean ± SEM (n = 3) based on plasma membrane permeability elevation. *, P < 0.05; **, P < 0.01 vs control untreated cells.

B: FCM using FITC-Annexin V/propidium iodide (PI) staining. The cells were treated with the indicated exposure time of 0.3% OFX. Values are expressed as mean ± SEM (n = 3) based on phosphatidylserine (PS) externalization. *, P < 0.05; **, P < 0.01 vs control untreated cells.

C: DNA electrophoresis. The dosage and exposure time of OFX are shown on the top of each lane. Marker, D2000 standard molecular weight marker.

D: Transmission electron microscopy. The dosage and exposure time of OFX are shown in the top of each photograph. Scale bar, 1 μm. a, apoptotic body; mv, microvillus; N, nucleus; v, vacuole. m, mitochondrion.

**OFX induces MTP disruption of HCEP cells:** As well known, induction of apoptosis requires the cooperation of signal molecules and the key proteins converged on the membrane of the mitochondrion [36,37], and the disruption of MTP is a prerequisite for triggering mitochondrial release of cytochrome c, AIF and Smac/Diablo [36,38,39]. To confirm whether a mitochondrion signaling pathway was involved in OFX-induced apoptosis, we further investigated the MTP disruption. Our results of FCM using JC-1 staining found that the number of JC-1 positive cells (MTP-disrupted cells) increased to 14.11 ± 0.27% (P < 0.01), 28.19 ± 0.35% (P < 0.01), and 75.09 ± 0.34% (P < 0.01) after treated with 0.3% OFX for 8h, 12h, and 16h, respectively (Figure 4). These demonstrate that OFX could induce MTP disruption in HCEP cells, implying a mitochondrion dependent apoptotic pathway might be involved in OFX-induced apoptosis [22].

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**Figure 3**: OFX induces caspase activation in human corneal epithelial cells. The cells were treated with 0.3% OFX for the indicated exposure time. Values are expressed as mean ± SEM (n = 3) based on ELISA using the active forms of caspase-2, -3, and -9 antibodies. *, P < 0.05; **, P < 0.01 vs control untreated cells.

**Figure 4**: OFX induces mitochondrial transmembrane potential (MTP) disruption of human corneal epithelial cells. The cells were treated with 0.3% OFX for the indicated exposure time. MTP of the cells was detected by FCM using JC-1 staining. The JC-1 positive cells represent the cells with depolarized mitochondria. Values are expressed as mean ± SEM (n = 3). *, P < 0.05; **, P < 0.01 vs control untreated cells.

**OFX induces altered expression of apoptotic proteins in HCEP cells**: As well defined, subcellular distribution and the ratio of pro- vs anti-apoptotic Bcl-2 family members localized at the mitochondrion is a critical determinant in the ability of cells to undergo apoptosis [40]. To determine the molecular mechanisms of OFX-induced apoptosis, we further examined the expression of Bcl-2 family proteins and mitochondrion released proteins in HCEP cells by Western blot. Our results showed that the expression level of Bcl-2 and Bcl-xL was down-regulated, while that of Bax, Bad, cytochrome c and AIF was up-regulated in HCEP cells after exposed to 0.3% OFX for 6 h and 12 h, respectively (P < 0.05 or 0.01) (Figure 5).

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As we know, the anti-apoptotic members of Bcl-2 and Bcl-xL function as a gatekeeper of mitochondria to prevent the release of both cytochrome c and AIF, while the pro-apoptotic members of Bax and Bad interact with the permeability transition pore to induce mitochondrial permeability transition and cytochrome c release from mitochondrion into the cytoplasm [40-42]. Our results of the down-regulation of Bcl-2 and Bcl-xL and the up-regulation of Bax and Bad suggest that the OFX induces apoptosis in HCEP cells through altered expression of Bcl-2 family proteins. As well postulated in mitochondrion-dependent apoptosis, cytochrome c is an important mediator in apoptotic pathways by activating procaspase-9 in cooperation with Apaf-1 after released from mitochondrion [43], and AIF is an apoptosis-inducing flavoprotein endowed with the unique capacity to induce caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation by activating Poly (ADP-ribose) polymerase-1 [44-46]. The up-regulation of cytochrome c and AIF, combined with caspase-9 activation, indicates that the OFX-induced HCEP cell apoptosis is regulated by a mitochondrion-dependent signaling pathway. This is supported by the previous reports on the apoptosis triggered by chemotherapeutic agents [18,22,24,47,48].

To our knowledge, this is the first attempt of studying the cytotoxicity of OFX to HCEP cells and its cytotoxic mechanisms at cellular and molecular levels in vitro. This model has been proven to be a rapid and cost-effective method to screen for surface toxicity of topical antibiotics, and our findings provide new insights into the non-negligible cytotoxicity and apoptosis-inducing effect of OFX on HCEP cells. Even these findings are particularly relevant in deciding the optimal fluoroquinolone antibiotic to be applied in clinical situations; they do not allow predicting clinical inferences directly without further investigations in vivo. However, the dose- and time-dependent cytotoxicity of OFX on HCEP cells reveals that this topical antibiotic should be used with great caution in eye clinic and its repeated and prolonged usage should be prohibited.

Conclusion

In summary, OFX above 1/8 of its therapeutic concentration has a dose- and time-dependent cytotoxicity to HCEP cells in vitro, and its cytotoxicity is realized by inducing cell cycle arrest and apoptosis which is regulated by a death receptor-mediated mitochondrion-dependent signaling pathway. Our findings provide new insights into the non-negligible cytotoxicity and apoptosis-inducing effect of OFX on human corneal cells.

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Conflict of Interest

The authors certify that no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest exists.

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