Cytotoxicity of atropine to human corneal endothelial cells by inducing mitochondrion-dependent apoptosis

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Abstract

Atropine, a widely used topical anticholinergic drug, might have adverse effects on human corneas *in vivo*. However, its cytotoxic effect on human corneal endothelium (HCE) and its possible mechanisms are unclear. Here, we investigated the cytotoxicity of atropine and its underlying cellular and molecular mechanisms using an *in vitro* model of HCE cells and verified the cytotoxicity using cat corneal endothelium (CCE) *in vivo*. Our results showed that atropine at concentrations above 0.3125 g/L could induce abnormal morphology and viability decline in a dose- and time-dependent manner *in vitro*. The cytotoxicity of atropine was proven by the induced density decrease and abnormality of morphology and ultrastructure of CCE cells *in vivo*. Meanwhile, atropine could also induce dose- and time-dependent elevation of plasma membrane permeability, G1 phase arrest, phosphatidylserine externalization, DNA fragmentation, and apoptotic body formation of HCE cells. Moreover, 2.5 g/L atropine could also induce caspase-2/-3/-9 activation, mitochondrial transmembrane potential disruption, downregulation of anti-apoptotic Bcl-2 and Bcl-xL, upregulation of pro-apoptotic Bax and Bad, and upregulation of cytoplasmic cytochrome c and apoptosis-inducing factor. In conclusion, atropine above 1/128 of its clinical therapeutic dosage has a dose- and time-dependent cytotoxicity to HCE cells *in vitro* which is confirmed by CCE cells *in vivo*, and its cytotoxicity is achieved by inducing HCE cell apoptosis via a death receptor-mediated mitochondrion-dependent signaling pathway. Our findings provide new insights into the cytotoxicity and apoptosis-inducing effect of atropine which should be used with great caution in eye clinic.

Keywords: Atropine, human corneal endothelial cells, cat corneal endothelial cells, cytotoxicity, apoptosis, mitochondrion

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Introduction

Human corneal endothelium (HCE) is a single layer of hexagonal cells, which plays vital roles in maintaining corneal transparency and thickness by regulating stromal hydration.¹ Once a mature HCE monolayer has formed, cell proliferation ceases and cell density continues to decrease throughout life at an average rate of 0.3%–0.6% per year.² Cell enlargement and migration are the major means of monolayer repair, and excessive loss of HCE cells, on account of dystrophy, surgical trauma, or disease, can result in decompensation of the corneal endothelium which will lead to loss of visual acuity, corneal edema, and loss of vision eventually.¹ Therefore, it will be of great value to identify the cytotoxic effects of ophthalmic drugs in use on HCE cells.

Atropine is often used for mydriasis in optometry, paralysis in cataract surgery, and paralysis in penalization treatment of functional amblyopia in eye clinic.^{3–6} It is indicated in recent researches that atropine might have adverse

effects on human eyes, such as dilated pupils, blurred vision, and light sensitivity, and which compromise visual acuity.^{7,8} However, whether atropine has cytotoxic effects on HCE cells is still a mystery. One of the obstacles in studying intensively the cytotoxicity of atropine is the lack of an *in vitro* model of HCE cells that can be used to investigate the possible cytotoxic mechanisms and the prospective therapeutic interventions. Although Simian Virus 40-immortalized HCE cell line was established and used for *in vitro* studies previously,^{9,10} their validity in endothelial cell studies has been greatly limited due to its genetic instability, abnormal phenotype, and tumorigenic potency.¹¹ Recently, an established non-transfected HCE cell line, with a normal genotype and inherent properties along with a normal phenotype in corneal equivalent construction,^{12,13} make it possible to study the cytotoxicity of atropine on HCE cells *in vitro* and its possible cellular and molecular mechanisms as well.¹⁴ The present study was intended to investigate the cytotoxicity of atropine to HCE cells *in vitro*, verify the

cytotoxicity using an *in vivo* model of cat corneas,¹⁵ and reveal the cytotoxic mechanisms using an *in vitro* model of non-transfected HCE cells.

Materials and methods

Test chemical

Atropine (Sigma-Aldrich, St. Louis, MO, USA) was first dissolved into serum-free Dulbecco's modified Eagle medium: Ham's nutrient mixture F-12 (DMEM/F12) (1:1) medium (Invitrogen, Carlsbad, CA, USA) to prepare a 80 g/L stock solution before usage, and double-diluted with 20% (v/v) fetal bovine serum (FBS) (Invitrogen)-DMEM/F12 medium to a final concentration from 40 g/L to 0.15625 g/L.

Experimental animals and housing conditions

Four male domestic cats, weighting of 2.0–2.5 kg, were provided by the Animal Center of Qingdao Chunghao Biotech Company (Qingdao, China) and acclimated for one week prior to the commencement of the experiment. They were maintained in an air-conditioned animal room with a temperature of $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$, a relative humidity of $55\% \pm 5\%$, ventilation frequency of 18 times per hour, and a 12-h light/dark cycle. Each cat was housed in isolated stainless steel cages and allowed free access to water and food throughout the acclimation period. All experimental procedures using animals were approved by the ethics review board of the company. Animal protocols were in adherence to the guidelines in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell culture and atropine treatment

HCE cells, from the non-transfected HCE cell line (nHCEC01) established previously in our laboratory,¹² were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37°C in 25 cm^2 flasks (Nunc, Copenhagen, Denmark), and harvested by 0.25% trypsin digestion (1 min) and centrifugation (120 g, 10 min) as described previously.¹⁴ Once the cells proliferated into logarithmic phase, the culture medium was replaced entirely with fresh medium containing atropine at concentrations ranging from 40 g/L (the therapeutic dosage in eye clinic) to 0.15625 g/L and cultured as described above. HCE cells cultured in the same medium without any atropine addition at the same time point were used as controls in all experiments.

Light microscopy

The growth and morphology of HCE cells were monitored by light microscopy as described previously.¹⁴ Briefly, HCE cells were inoculated into a 24-well culture plate (Nunc) and cultured in 10% (v/v) FBS-DMEM/F12 medium at 37°C in a humidified 5% CO_2 incubator. Logarithmic HCE cells were treated with atropine at concentrations from 0.15625 g/L–40 g/L as described above. The cells were

cultured under the same condition as described above, and their growth status and morphology were monitored every 4 h under an Eclipse TS100 inverted light microscope (Nikon, Tokyo, Japan).

Methyl thiazolyl tetrazolium (MTT) assay

Cell viability of HCE cells was measured by MTT assay as described previously.¹⁴ In brief, HCE cells were inoculated into 96-well culture plates (Nunc) at a density of 1×10^4 cells per well and were cultured and treated as described above. After atropine treatment, the culture medium of the cells was replaced entirely with 200 μL serum-free DMEM/F12 medium containing 1.1 mM MTT (Sigma-Aldrich) every 1 to 4 h and then incubated 4 h at 37°C in the dark. After, the MTT-containing medium was replaced with 150 μL of dimethyl sulfoxide (Sigma-Aldrich) to dissolve the produced formazan. The 490 nm absorbance of each well was measured with a Multiskan GO microplate reader (Thermo Scientific, MA, USA).

In vivo detection of cat corneal endothelial cells

The cytotoxicity of atropine to corneal endothelial cells was verified using the *in vivo* model of cat corneas as described previously.¹⁴ The corneas of the right eyes of four cats, with similar cat corneal endothelium (CCE) cell density, were squeezed with two drops of 40 g/L atropine (three times daily for seven consecutive days), and all corneas of the left eyes were used as normal controls. The corneal endothelial cell density (ECD) was monitored by a SP-3000P specular microscope (Topcon, Tokyo, Japan) every five days. Besides, accessory central corneal thickness (CCT) and intraocular pressure of cat eyes was also detected using a 300P ultrasound pachymeter (Sonomed, NY, USA) and a Reichert Tono-Pen AVIA applanation tonometer (Nikon), respectively. Thirty days later, the cats were all euthanized with ether and their corneas were sampled for *ex vivo* examination. The cornea endothelium from each cat was examined by 1% alizarin red staining, scanning electron microscopy (JSM-840; JEOL, Tokyo, Japan), and transmission electron microscopy (TEM; H700, Hitachi, Tokyo, Japan) as described previously.¹³

Double fluorescent staining

Plasma membrane permeability of HCE cells was examined by acridine orange (AO)/ethidium bromide (EB) double-fluorescent staining according to the method described previously.¹⁶ Briefly, HCE cells in a 24-well culture plate (Nunc) were cultured, treated with atropine and harvested as described above. After the cells were stained with 4 μL of 100 mg/L AO/EB (Sigma-Aldrich) solution (AO: EB = 1:1) for 1 min at room temperature, the cell suspension from each group was observed under a Ti-S fluorescent microscope (Nikon). HCE 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 as 'Apoptotic cells/(Apoptotic cells + Non-apoptotic cells) $\times 100$ ' with at least 400 cells counted in each group.

DNA agarose gel electrophoresis

DNA fragmentation of HCE cells was detected by agarose gel electrophoresis as described previously.¹⁶ In brief, HCE cells in 25 cm² flasks were cultured, treated, and harvested as described above. After the cells were washed once with ice-cold phosphate-buffered saline (PBS) by centrifugation (200 g, 10 min), their DNA was extracted with a Quick Tissue/Culture Cells Genomic DNA Extraction Kit (Dongsheng Biotechnology, Guangzhou, China) following the manufacturer's instructions. The DNA sample from each group was electrophoresed in 1% (w/v) agarose gel (200 mA, 260 min) together with the D2000 standard molecular weight marker (Takara Bio Inc., Ishiyama, Japan), stained with 0.5 mg/L EB for 10 min, and observed with an EC3 Imaging System (UVP, LLC Upland, CA, USA).

Transmission electron microscopy (TEM)

The ultrastructure of HCE cells was characterized by TEM following the method reported previously.¹⁴ Briefly, HCE cells in 25 cm² flasks were cultured, treated with 2.5 g/L atropine, and harvested as described above. After fixed successively with 4% (v/v) glutaraldehyde and 1% (v/v) osmium tetroxide, the cells were dehydrated and embedded in epoxy resin. Ultrathin sections were stained with 2% (w/v) uranyl acetate-lead citrate and observed with an H700 TEM (Hitachi).

Flow cytometry (FCM)

The cell cycle progression, phosphatidylserine (PS) externalization, and mitochondrial transmembrane potential (MTP) of HCE cells were detected and analyzed by flow cytometry as reported previously.¹⁴ In brief, HCE cells in a 24-well culture plate (Nunc) were cultured, treated with 2.5 g/L atropine, and harvested as described above. After washed once with 1 mL PBS by centrifugation (200 g, 5 min), the cells were fixed with 70% (v/v) alcohol at 4°C overnight. Then the fixed cells were stained with propidium iodide (PI) (BD Biosciences, San Jose, CA, USA) for cell cycle assay, stained with fluorescein isothiocyanate (FITC)-labeled annexin-V/PI using a FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) for PS externalization assay, and stained with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide) (Sigma-Aldrich) for MTP assay, respectively. The stained HCE cells were detected by a FACScan flow cytometer and analyzed with CXP analysis software (BD Biosciences).

ELISA for caspase activation

Caspase activation of HCE cells was measured by ELISA as described previously.¹⁷ Briefly, HCE cells in six-well plates (Nunc) were cultured, treated with 2.5 g/L atropine, and harvested every 2 h as described above. Total Whole-cell protein extracts were prepared using 500 µL radio-immunoprecipitation assay (RIPA) lysis buffer (CWbiotech, Beijing, China) and coated into a high binding 96-well microtiter plates (Nunc) at 4°C overnight. Following three washes with PBS containing 0.05% Tween-20 (PBST), the wells were blocked with 5% non-fat milk (BD Bioscience) at 37°C

for 2 h. Following three washes with PBST, the wells were incubated with 100 µL rabbit anti-human caspase-2, -3, and -9 (active form) antibodies (1:500) (Santa Cruz, CA, USA) at 37°C for 2 h, and 100 µL horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:3000) (Santa Cruz) at 37°C for 1 h, respectively. Following three-washes, a colorimetric reaction was induced by 100 µL chromogenic substrate (0.1 mg/mL tetramethylbenzidine, 100 mM acetate buffer, pH 5.6, and 1 mM urea hydrogen peroxide) for 25 min at room temperature in the dark. Colour development was stopped with 50 µL H₂SO₄ (0.5 M), and the 450 nm absorbance of each well was measured using a Multiskan GO microplate reader (Thermo Scientific).

Western blot assay

The expression of Bcl-2 family proteins and cytoplasmic amount of mitochondrion-released apoptotic proteins were assayed by Western blot. HCE cells were cultured, treated with 2.5 g/L atropine, and harvested every 4 h as described above. Whole-cell protein extracts were prepared using RIPA lysis buffer (CWbiotech), and cytoplasmic protein extracts were prepared using a mitochondria and cytoplasmic protein extraction kit (Sangon biological engineering, Shanghai, China), respectively. The protein extract from the same number of cells in each group was electrophoresed by 10% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocked with 50 g/L nonfat milk at room temperature for 1 h, the membranes were incubated with rabbit anti-human IgG monoclonal antibody to β-actin (1:1000), Bcl-2 (1:1000), Bcl-xL (1:10000), Bax (1:2000), Bad (1:2000), cytochrome c (Cyt. c, 1:5000), and apoptosis-inducing factor (AIF, 1:1000) (all from Abcam, Cambridge, UK), respectively, at 4°C overnight. Following three washes with PBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG monoclonal antibody (1:2000, abcam) at room temperature for 1.5 h. Finally, the protein bands were visualized using a cECL Western Blot Kit (CWbiotech) following the manufacturer's instructions and were observed under an EC3 Imaging System (UVP). The optical density of each band was quantified using Quantity One 4.4.0 image analysis software (Bio-Rad, CA, USA) with β-actin as an internal control.

Statistical analysis

Experiments were repeated three times (*in vitro*) or four times (*in vivo*) independently. All data were presented as mean ± SD and analyzed for statistical significance with Student's *t*-test using SPSS 17.0 software (SPSS Inc., IL, USA). Differences to controls were considered statistically significant when *P* < .05.

Results

Growth retardation and morphological abnormality of HCE cells

Light microscopic observations showed that *in vitro*-cultured HCE cells treated with atropine at concentrations ranging from 0.3125 g/L to 40 g/L exhibited growth

retardation and abnormal morphological changes similar to those of apoptotic cells including cytoplasmic vacuolation, cellular shrinkage, detachment from culture matrix, and eventually death, in a dose- and time-dependent manner, while those treated with 0.15625 g/L atropine showed no obvious difference to controls (Figure 1).

Cell viability decline and cell cycle arrest of HCE cells

MTT assay showed that the cell viability of *in vitro*-cultured HCE cells decreased with concentration and time-dependent after exposed to atropine above the concentration of 0.3125 g/L (1/128 of its clinical therapeutic dosage) ($P < .01$), while that of HCE cells treated with atropine below the concentration of 0.3125 g/L showed no significant difference to controls (Figure 2(a)). Moreover, results of FCM using propidium iodide (PI) staining indicated that the number of 2.5 g/L atropine-treated HCE cells in G1 phase increased with time, while that of HCE cells in S phase decreased with time (Figure 2(b)), implying that atropine could induce viability decline and G1 phase arrest of HCE cells.

Toxic effect of atropine on CCE cells in vivo

Results of specular microscopy showed that the ECD of CCE cells of cat eyes decreased with time ($P < .01$ or 0.05) after exposed to 40 g/L atropine *in vivo* (Figure 3(a)), while the corneal thickness showed no significant difference to controls during the monitoring period (Figure 3(b)). The increased of IOP was only found within the first 10 days

post atropine exposure (Figure 3(c)). *Ex vivo* characterization of cat corneas 30 days after exposed to atropine was summarized in Figure 3(d). Results of alizarin red staining and SEM displayed that a lot of apoptotic-like swollen CCE cells (arrow heads in Figure 3(d)) appeared in the corneal endothelia of atropine-treated cat eyes. Moreover, TEM observation indicated that CCE cells from corneas exposed to atropine underwent mitochondrial swelling (arrows in Figure 3(d)). These results suggest that atropine at its clinical therapeutic dosage has significant cytotoxicity to CCE cells *in vivo*.

Plasma membrane abnormality of HCE cells

The plasma membrane abnormality of HCE cells was estimated based on the PS externalization and permeability elevation. Results of FCM using Annexin V-FITC/PI staining showed that 2.5 g/L atropine could induce PS externalization in the plasma membrane of HCE cells in a time-dependent manner, and the number of atropine-induced apoptotic cells (Annexin V positive cells) increased to $28.69\% \pm 0.87\%$ ($P < .01$) at 2 h, $82.36\% \pm 1.27\%$ ($P < .01$) at 4 h, and $86.67\% \pm 1.62\%$ ($P < .01$) at 8 h, respectively, when compared with its corresponding control (Figure 4(a)). AO/EB double staining showed that atropine could also induce dose- and time-dependent elevation of plasma membrane permeability of HCE cells at concentrations above 0.3125 g/L ($P < .01$ or .05). While no significant difference was found in HCE cells exposed to 0.15625 g/L atropine when compared with the control (Figure 4(b)). All these indicate that

Figure 1 Growth status and morphological changes of atropine-treated HCE cells. Cultured HCE cells were treated with the indicated concentration and exposure time of atropine, and their growth and morphology were monitored by light microscopy. One representative photograph from three independent experiments was shown

atropine at concentrations above 0.3125 g/L could result in plasma membrane abnormality of HCE cells by inducing PS externalization and permeability elevation.

DNA fragmentation and ultrastructural abnormality of HCE cells

The results of agarose gel electrophoresis indicated that the DNA extracted from atropine-treated HCE cells was fragmented into a highly dispersed state, and typical DNA ladders appeared in electrophoresed gels in a dose- and

time-dependent manner, while no DNA ladder was found in control cells (Figure 5(a)). TEM observations showed that 2.5 g/L atropine-treated HCE cells exhibited apoptosis-like ultrastructural changes, such as microvillus disappearance, cytoplasmic vacuolation, structural disorganization, mitochondrion swelling, chromatin condensation, and apoptotic body formation, when compared with untreated control cells (Figure 5(b)). These suggest that atropine could induce DNA fragmentation and apoptosis-like ultrastructural abnormality of HCE cells.

Figure 2 Cell viability decline and cell cycle arrest of atropine-treated HCE cells. Cultured HCE cells were treated with the indicated concentration and exposure time of atropine. (a) MTT assay. The cell viability of atropine-treated HCE cells in each group was expressed as percentage (mean $\pm$ SD) of 490 nm absorbance compared to its corresponding control ($n = 3$). b, $P < .01$ vs control. (b) FCM by PI staining. One representative image from three independent experiments was shown. The cell number of HCE cells in each phase of the cell cycle was expressed as percentage of the total number of cells

Figure 3 *In vivo* examination of 40 g/L atropine-exposed cat eyes. (a) Endothelial cell density (ECD) of CCE cells. (b) Central corneal thickness (CCT) of cat corneas. (c) Intraocular pressure (IOP) of cat eyes. Data in (a), (b), and (c) were all expressed as mean $\pm$ SD ($n = 4$). a, $P < .05$, b $P < .01$ vs normal control. (d) *Ex vivo* microscopic photographs of CCE cells 30 days after exposed to atropine. One representative photograph from four cats was shown. The swollen cells in alizarin red staining and SEM images were indicated by arrow heads, and swollen mitochondria in TEM images were indicated by arrows. SEM: scanning electron microscopy; TEM: transmission electron microscopy

Figure 4 PS externalization and plasma membrane permeability elevation of atropine-treated HCE cells. Cultured HCE cells were treated with the indicated concentration and exposure time of atropine and their plasma membrane abnormality were assayed. (a) FCM by Annexin V/PI staining. Annexin V⁺ cells (PS-externalized cells) were identified as apoptotic cells. The cell number of HCE cells in each group was expressed as percentage (mean $\pm$ SD) of the total number of cells ($n = 3$). a, $P < .05$, b $P < .01$ vs control. (b) AO/EB double staining. The apoptotic ratio was calculated as percentage (mean $\pm$ SD) of the total number of cells based on the permeability elevation of plasma membrane of HCE cells ($n = 3$). a, $P < .05$, b $P < .01$ vs control

Figure 5 DNA fragmentation and ultrastructural abnormality of atropine-treated HCE cells. Cultured HCE cells were treated with the indicated concentration and exposure time of atropine. (a) DNA electrophoresis. DNA isolated from HCE cells was electrophoresed in 1% (w/v) agarose gel, and dispersed DNA ladders were shown. (b) TEM photographs. One representative photograph from three independent experiments was shown. N: nucleus; RER: rough endoplasmic reticulum; chn: condensed chromatin; m: swollen mitochondrion; mv: microvilli; v: vacuole; *: apoptotic body

Caspase activation and MTP disruption in HCE cells

Results of ELISA showed that caspase-2, -3, and -9 from 2.5 g/L atropine-treated HCE cells were all activated successively ($P < .01$ or $.05$), of which caspase-2 was first activated to its peak value at 4 h ($P < .01$), caspase-9 to its peak value at 6 h ($P < .01$), and caspase-3 to its peak value at 10 h ($P < .01$) (Figure 6(a)). FCM with JC-1 staining revealed that 2.5 g/L atropine could induce MTP disruption in HCE cells, and the number of monomer JC-1 positive cells (MTP-disrupted cells) increased in a time-dependent manner (Figure 6(a)). This indicates that atropine can induce MTP disruption of HCE cells; 6.25 mg/L latanoprost could induce MTP disruption in HCE cells, and the number of JC-1 positive cells (MTP-disrupted cells) increased to $27.4\% \pm 0.3\%$ ($P < .01$) and $45.6\% \pm 0.4\%$ ($P < .01$) at 6 h and 12 h, respectively, after latanoprost exposure

Altered expression and translocation of apoptotic proteins in HCE cells

Results of Western blot displayed that the expression level of anti-apoptotic Bcl-2 family proteins of Bcl-2 and Bcl-xL was down-regulated, while that of pro-apoptotic Bcl-2 family proteins of Bax and Bad was up-regulated, and the

cytoplasmic amount of mitochondrion-released proteins of Cyt. c and AIF was up-regulated in 2.5 g/L atropine-treated HCE cells in a time-dependent manner (Figure 7).

Discussion

Damages from topical drugs and trauma to the cornea, especially to corneal endothelium, often result in excessive loss of HCE cells which will lead to decompensation of corneal endothelium and visual impairment.¹ Atropine, a frequently used anticholinergic drug in eye clinic, was reported to have adverse effects on human eyes.^{7,8} However, little has been known about the effect of atropine on the cornea. In order to provide a reference for secure medication of atropine, the present study was intended to evaluate the cytotoxicity of atropine to HCE cells using an *in vitro* model of non-transfected HCE cells, verify the cytotoxicity using an *in vivo* model of cat corneas, and clarify its possible cellular and molecular cytotoxic mechanisms *in vitro*.

To characterize the cytotoxicity of atropine, the morphology, viability and cell cycle status of HCE cells were investigated at first *in vitro*. Our results revealed that atropine at

Figure 6 Caspase activation and MTP disruption in atropine-treated HCE cells. Cultured HCE cells were treated with 2.5 g/L atropine for the indicated exposure time. (a) ELISA detection. Caspase activation was measured by ELISA using monoclonal antibodies against the active form of caspase-2, -3, and -9. The activation ratio of each group was expressed as percentage (mean $\pm$ SD) compared to its corresponding control based on the 450 nm absorbance ($n=3$). a, $P < .05$; b, $P < .01$ vs control. (b) FCM using JC-1 staining. JC-1 in mitochondria with MTP depolarized maintains monomers in green fluorescence, while that in mitochondria with normal MTP incorporates into aggregates in red fluorescence. The number of JC-1 positive cells (MTP-disrupted cells) in each group was expressed as percentage (mean $\pm$ SD) of the total number of cells ($n=3$). b, $P < .01$ vs control

Figure 7 Altered expression of Bcl-2 family proteins and cytoplasmic translocation of Cyt. c, and AIF in atropine-treated HCE cells. Cultured HCE cells were treated with 2.5 g/L atropine for the indicated exposure time, and the expression level of Bcl-2 family proteins and mitochondrion release of apoptotic proteins were examined by western blotting. (a) Western blot images. (b) Densitometry analyses. The relative level of protein expression was expressed as percentage (mean $\pm$ SD) of protein band density compared to the internal control of β -actin ($n=3$). b, $P < .01$ vs control

concentrations above 0.3125 g/L (1/128 of its clinical therapeutic dosage) could induce growth retardation, apoptotic-like morphological changes, and viability decline of HCE cells in a dose- and time-dependent manner. Meanwhile, 2.5 g/L atropine could also induce G1 phase arrest of HCE cells. All these suggest that atropine above 1/128 of its therapeutic dosage has notable cytotoxicity to HCE cells *in vitro*, and G1 phase arrest might be involved in atropine-induced proliferation retardation of HCE cells. These findings are supported by previously reported cytotoxicity of topical drugs and anesthetics on HCE cells.^{14,16-18}

Even these findings are particularly relevant in deciding the cytotoxicity of atropine, they do not allow predicting clinical inferences directly without *in vivo* investigations. To verify the cytotoxic effect of atropine, an *in vivo* model of CCE was used in the present study. Our results showed that atropine at its clinical therapeutic dosage of 40 g/L

could induce time-dependent reduction of ECD of CCE cells, and apoptotic-like morphological and ultrastructural changes, including cell swelling and mitochondrial swelling in CCE cells *in vivo*. In general, mitochondrial swelling due to the opening of permeability transition pores is often associated with apoptosis.¹⁹ These results suggest that atropine has a time-dependent cytotoxicity to CCE cells *in vivo* by inducing mitochondrial damage and perhaps apoptosis.

Our results of the dose- and time-dependent cytotoxicity of atropine to HCE cells *in vitro*, combined with the time-dependent cytotoxicity to CCE cells *in vivo*, indicate that atropine do have a notable cytotoxicity to corneal endothelial cells, which is also supported by our previously reported cytotoxicity of betaxolol and proparacaine on corneal endothelial cells.^{9,16}

Since cell viability decline, cell cycle arrest and mitochondrial swelling are often related with apoptosis triggered by chemical agents.^{14,17,20,21} Our results suggest that the

cytotoxicity of atropine to HCE cells might be related with apoptosis.

To verify whether the cytotoxicity of atropine is achieved by inducing apoptosis, PS externalization, plasma membrane permeability elevation, DNA fragmentation, and apoptotic body formation of atropine-treated HCE cells were examined *in vitro*, because these indexes are the hallmark features of apoptosis.^{22–24} We found that atropine above concentrations of 0.3125 g/L could induce dose- and time-dependently the membrane permeability elevation and DNA fragmentation of HCE cells, and 2.5 g/L atropine could also induce PS externalization, ultrastructural disorganization, cytoplasmic vacuolation, mitochondrial swelling, chromatin condensation, and apoptotic body formation. As well known, 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 main characteristic difference of apoptosis from necrosis.²⁵ All these verify that atropine could induce apoptosis of HCE cells *in vitro*. These findings are also supported by our previous reports on the apoptosis-inducing effects of topical drugs and anesthetics on HCE cells.^{14,16–18}

Generally, apoptosis is a normal physiological process which can be triggered via two main signaling pathways, the death receptor-mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway,²⁶ both of them result in activation of initiator caspases (caspase-2, -8, -10, and -9) and the subsequent executioner caspases (caspase-3, -6, and -7).²⁷ To determine the possible apoptosis-triggering pathway of atropine-induced apoptosis, we further detected the activation pattern of caspase-2, -3, and -9 by ELISA using the monoclonal antibodies of their active forms. Our results indicated that 2.5 g/L atropine could activate caspase-2, -9 and -3 hierarchically in HCE cells. Since caspase-2 is a key mediator of tumor necrosis factor receptor 1 (TNFR1)-mediated extrinsic pathway while caspase-9 is a key regulator of the mitochondrion-dependent intrinsic pathway.^{27,28} Our results of the activation of caspase-2, -9 and -3 in atropine-treated HCE cells suggest that the atropine-induced HCE cell apoptosis might be triggered via both a death receptor-mediated and a mitochondrion-dependent pathway. The involvement of both an extrinsic and an intrinsic signaling pathways has also been reported in other chemical induced apoptosis.^{14,17,29–31}

As well elucidated, the regulation of apoptosis requires the concordant cooperation of caspases, pro-apoptotic Bcl-2 family proteins (including Bax and Bad), anti-apoptotic Bcl-2 family proteins (including Bcl-2 and Bcl-xL), and the other mitochondrion-sequestered apoptosis-triggering proteins, and disruption of MTP is a prerequisite for triggering mitochondrial release of Cyt. c (an indispensable activator of caspase-9), AIF (an pivotal inducer of caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation), and Smac/Diablo (a pro-apoptotic protein to increase caspase activation by inhibiting the inhibitor of apoptosis proteins).^{27,32–34}

To confirm the involvement of a mitochondrion signaling pathway in atropine-induced apoptosis, we also investigated MTP disruption, the expressional changes of Bcl-2

family proteins, and the cytoplasmic translocation of Cyt. C, and AIF. Our results displayed that atropine could induce MTP disruption, down-regulation of Bcl-2 and Bcl-xL expression, up-regulation of Bax and Bad expression, and up-regulation of the mitochondrial release of Cyt. c and AIF in cytoplasm, respectively. As well known, anti-apoptotic Bcl-2 and Bcl-xL function as a gatekeeper of the mitochondrion to prevent the release of mitochondrial Cyt. c and AIF, while the pro-apoptotic Bax and Bad induce the release of Cyt. c and AIF into cytoplasm by interacting with the mitochondrion permeability transition pore.^{35–37} These results, combined with caspase-2 and -9 activation, indicate that the apoptosis-inducing effect of atropine on HCE cells is most probably regulated by a Bcl-2 family protein-involved and death receptor-mediated mitochondrion-dependent signaling pathway.

In summary, atropine above 1/128 of its clinical therapeutic concentration has a dose- and time-dependent cytotoxicity to HCE cells *in vitro*, and the cytotoxicity is confirmed by CCE cells *in vivo*. The cytotoxicity of atropine to HCE cells is achieved by inducing death receptor-mediated mitochondrion-dependent apoptosis. To our knowledge, this is the first attempt of investigating the cytotoxicity of atropine to HCE cells and its cytotoxic mechanisms at cellular and molecular levels *in vitro*, and these findings provide new insights into the acute cytotoxicity and apoptosis-inducing effect of atropine on HCE cells. Even our findings do not allow predicting clinical inferences on the adverse effect of atropine directly, they reveal that atropine should be used with great caution in eye clinic.

Author contributions: QW performed the research, data analysis, and interpretation and prepared the manuscript. TJF was involved in conception and design, data analysis and interpretation, and review of the manuscript. CLT assisted in the research related to flow cytometry and the statistical analyses.

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DECLARATION OF CONFLICTING INTEREST

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REFERENCES

- Joyce NC. Proliferative capacity of the corneal endothelium. *Prog Retin Eye Res* 2003;22:359–89
- Hollingsworth J, Perez-Gomez I, Mutalib HA, Efron N. A population study of the normal cornea using an in vivo slit-scanning confocal microscope. *Optom Vis Sci* 2001;78:706–11
- Arnold RW, Gionet E, Hickel J, Owen M, Armitage MD. Duration and effect of single-dose atropine: paralysis of accommodation in penalization treatment of functional amblyopia. *Binocul Vis Strabismus Q* 2004;19:81–6

4. Lu PC, Chen JC. Retarding progression of myopia with seasonal modification of topical atropine. *J Ophthalmic Vis Res* 2010;**5**:75–82
5. Chia A, Chua WH, Cheung YB, Wong WL, Lingham A, Fong A, Tan D. Atropine for the treatment of childhood myopia: safety and efficacy of 0.5%, 0.1%, and 0.01% doses (Atropine for the Treatment of Myopia 2). *Ophthalmology* 2012;**19**:347–54
6. Cooper J, Eisenberg N, Schulman E, Wang FM. Maximum atropine dose without clinical signs or symptoms. *Optom Vis Sci* 2013;**90**:1467–72
7. Arendse R, Irusen E. An atropine and glycopyrrolate combination reduces mortality in organophosphate poisoning. *Hum Exp Toxicol* 2009;**28**:715–20
8. Bhattacharjee AK. Discovery of subtype selective muscarinic receptor antagonists as alternatives to atropine using in silico pharmacophore modeling and virtual screening methods. *Bioorg Med Chem* 2013;**21**:2651–62
9. Wilson SE, Lloyd SA, He YG, McCash CS. Extended life of human corneal endothelial cells transfected with the SV40 large T antigen. *Invest Ophthalmol Vis Sci* 1993;**34**:2112–23
10. Huang B, Blanco G, Mercer RW, Fleming T, Pepose JS. Human corneal endothelial cell expression of Na⁺, K⁺-adenosine triphosphatase isoforms. *Arch Ophthalmol* 2003;**121**:840–5
11. Cho KS, Song IK, Fleming TP, Joo CK. Proliferation of corneal endothelial cells by delivery of SV40 large T antigen. *Kor J Ophthalmol Soc* 2001;**42**:1331–5
12. Fan T, Zhao J, Ma X, Xu X, Zhao W, Xu B. Establishment of a continuous untransfected human corneal endothelial cell line and its biocompatibility to denuded amniotic membrane. *Mol Vis* 2011;**17**:469–80
13. Fan T, Ma X, Zhao J, Wen Q, Hu X, Yu H, Shi W. Transplantation of tissue-engineered human corneal endothelium in cat models. *Mol Vis* 2013;**19**:400–7
14. Wen Q, Fan TJ, Bai SR, Sui YL. Cytotoxicity of proparacaine to human corneal endothelial cells *in vitro*. *J Toxicol Sci* 2015;**40**:427–36
15. Van Horn DL, Sendele DD, Seideman S, Bucu PJ. Regenerative capacity of the corneal endothelium in rabbit and cat. *Invest Ophthalmol Vis Sci* 1977;**16**:597–613
16. Li Y, Wen Q, Fan T, Ge Y, Yu M, Sun L, Zhao Y. Dose dependent cytotoxicity of pranoprofen in cultured human corneal endothelial cells by inducing apoptosis. *Drug Chem Toxicol* 2015;**38**:16–21
17. Yu HZ, Li YH, Wang RX, Zhou X, Yu MM, Ge Y, Zhao J, Fan TJ. Cytotoxicity of lidocaine to human corneal endothelial cells *in vitro*. *Basic Clin Pharmacol Toxicol* 2014;**114**:352–9
18. Miao Y, Sun Q, Wen Q, Qiu Y, Ge Y, Yu MM, Fan TJ. Cytotoxic effects of betaxolol on healthy corneal endothelial cells both *in vitro* and *in vivo*. *Int J Ophthalmol* 2014;**7**:14–21
19. Rodriguez-Enriquez S, He L, Lemasters JJ. Role of mitochondrial permeability transition pores in mitochondrial autophagy. *Int J Biochem Cell Biol* 2004;**36**:2463–72
20. Lobner D. Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis? *J Neurosci Methods* 2000;**96**:147–52
21. Gogolin S, Ehemann V, Becker G, Brueckner LM, Dreidax D, Bannert S, Nolte I, Savelyeva L, Bell E, Westermann F. CDK4 inhibition restores G(1)-S arrest in MYCN-amplified neuroblastoma cells in the context of doxorubicin-induced DNA damage. *Cell Cycle* 2013;**12**:1091–104
22. Dallaporta B, Marchetti P, de Pablo MA, Maisse C, Duc HT, Métivier D, Zamzami N, Geuskens M, Kroemer G. Plasma membrane potential in thymocyte apoptosis. *J Immunol* 1999;**162**:6534–42
23. Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J Biol Chem* 2001;**276**:1071–7
24. Karmakar I, Halder S, Chakraborty M, Chaudhury K, Dewanjee S, Halder PK. Regulation of apoptosis through bcl-2/bax proteins expression and DNA damage by Zanthoxylum alatum. *Pharm Biol* 2015;**1**:1–6
25. White MK, Cinti C. A morphologic approach to detect apoptosis based on electron microscopy. *Methods Mol Biol* 2004;**285**:105
26. Matthews GM, Newbold A, Johnstone RW. Intrinsic and extrinsic apoptotic pathway signaling as determinants of histone deacetylase inhibitor antitumor activity. *Adv Cancer Res* 2012;**116**:165–97
27. Fan T, Han L, Cong R, Liang J. Caspase family proteases and apoptosis. *Acta Biochim Biophys Sin (Shanghai)* 2005;**37**:719–27
28. Wajant H. The Fas signaling pathway: more than a paradigm. *Science* 2002;**296**:1635–6
29. Fan WY, Wang DP, Wen Q, Fan TJ. Cytotoxicity of ofloxacin to human corneal epithelial cells by inducing cell cycle arrest and mitochondrion dependent apoptosis. *EC Ophthalmol* 2015;**1**:24–34
30. Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J Biol Chem* 1999;**274**:5053–60
31. Lee SA, Kim YJ, Lee CS, Brefeldin A induces apoptosis by activating the mitochondrial and death receptor pathways and inhibits focal adhesion kinase-mediated cell invasion. *Basic Clin Pharmacol* 2013;**113**:329–38
32. Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G. Mechanisms of cytochrome c release from mitochondria. *Cell Death Differ* 2006;**13**:1423–33
33. Cai J, Yang J, Jones DP. Mitochondrial control of apoptosis: the role of cytochrome c. *Biochim Biophys Acta* 1998;**1366**:139–49
34. Natarajan SK, Becker DF. Role of apoptosis-inducing factor, proline dehydrogenase, and NADPH oxidase in apoptosis and oxidative stress. *Cell Health Cytoskelet* 2012;**2012**:11–27
35. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Bio* 2014;**15**:49–63
36. Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, Tsujimoto Y. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. *Proc Natl Acad Sci USA* 1998;**95**:14681–6
37. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997;**275**:1132–36

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