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Cytotoxicity of atropine to human corneal epithelial cells by inducing cell cycle arrest and mitochondrion-dependent apoptosis

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ABSTRACT

Atropine is an anticholinergic drug for mydriasis in eye clinic, and its abuse might be cytotoxic to the cornea and result in blurred vision. However, the cytotoxicity of atropine to the cornea and its cellular and molecular mechanisms remain unknown. In this study, we investigated the cytotoxicity of atropine to corneal epithelium and its underlying mechanisms using an *in vitro* model of non-transfected human corneal epithelial (HCEP) cells. Our results showed that atropine, above the concentration of 0.3125 g/l (1/32 of its therapeutic dosage in eye clinic), had a dose- and time-dependent toxicity to HCEP cells by inducing morphological abnormality, cytopathic effect, viability decline, and proliferation retardation. Moreover, the proliferation-retarding effect of atropine on the cells was achieved by inducing G1/S phase arrest and downregulation of E-cadherin and β-catenin. Besides, atropine also had an apoptosis-inducing effect on the cells by inducing phosphatidylserine externalization, plasma membrane permeability elevation, DNA fragmentation and apoptotic body formation. Furthermore, atropine could also induce activations of caspase-2, -3 and -9, disruption of mitochondrial transmembrane potential, downregulation of Bcl-2 and Bcl-xL, upregulation of Bax and Bad, and upregulation of cytoplasmic cytochrome *c* and apoptosis-inducing factor, implying a death receptor-mediated mitochondrion-dependent pathway is most probably involved in the apoptosis of HCEP cells induced by atropine. Taken together, our results suggest that atropine has remarkable cytotoxicity to HCEP cells by inducing cell cycle arrest and death receptor-mediated mitochondrion-dependent apoptosis.

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1. Introduction

Human corneal epithelium (HCEP), a multi-layer structure located on the outermost of cornea, is crucial for maintaining a proper refractive surface for vision and a functional barrier for nutrient absorption, and ocular tissue protection as well (Kinoshita et al., 2001; Li, 2001). Physiologically, superficial HCEP cells undergo apoptosis regularly and their homeostasis are maintained by the proliferation and differentiation of limbal stem cells (Rama et al., 2010). However, damage of HCEP by drugs, infection and trauma often leads to HCEP cell deficiency which results in chronic inflammation, corneal neovascularization and even opacification (O'Callaghan and Daniels, 2011; Reim et al., 2001; Zhao and Panjwani, 1995).

Atropine, an anticholinergic drug, is widely used for mydriasis in optometry and paralysis in cataract surgery by influencing acetylcholine-induced contraction of smooth muscles (Drexler, 2010; Rabi et al., 2008; Siegling-Vlitakis, 2009). Unfortunately, atropine abuse often causes unexpected side effects on the cornea such as epithelial impairment and blurred vision (Bhattacharjee, 2013). Therefore, it will be of great importance to characterize the cytotoxicity of atropine to the cornea and the underlying cytotoxic mechanisms, especially to the outermost HCEP. However, little has been known about the cytotoxicity of atropine to HCEP due to the lack of an *in vitro* model of HCEP cells that can be used for cytotoxicity investigation. With the availability of a recently established non-transfected HCEP cell line (Fan et al., 2011), the present study was intended to investigate the toxic effect of atropine on HCEP cells and its underlying cytotoxic mechanisms *in vitro*.

2. Materials and methods

2.1. Material

HCEP cells from the established non-transfected HCEP cell line were cultured as described previously (Fan et al., 2011). Atropine powder with a purity ≥99% was purchased from Sigma–Aldrich (St. Louis, MO, USA). Before usage, 200 mg atropine powder was dissolved into serum-free DMEM medium (Gibco, NY, USA) to

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prepare a 20 g/l stock solution of atropine, and double-diluted with 20% (v/v) FBS-DMEM culture medium.

2.2. Light microscopy

The morphology and growth status were monitored by light microscopy. HCEP cells were inoculated into a 24-well culture plate (Nunc, Copenhagen, Denmark) and cultured in 10% (v/v) fetal bovine serum (FBS; Gibco)-DMEM medium at 37 °C in a 5% CO_2 incubator (Thermo Scientific, Waltham, MA, USA). Once the cells reached 80% confluence, the old medium in each well was completely replaced with 10–0.15625 g/l atropine solution diluted in 10% (v/v) FBS-DMEM medium, respectively. The cells were cultured under the same condition as described above, and their morphology and growth status were monitored successively with an Eclipse TS100 inverted microscope (Nikon, Tokyo, Japan) every 4 h.

2.3. MTT assay

The cell viability was detected by MTT assay as described previously (Yu et al., 2014). Briefly, HCEP cells were inoculated into a 96-well culture plate, and were cultured and treated with 0.15625–10 g/l atropine as described above. At a 1–4 h interval, 20 μl of 5 g/l MTT (Sigma–Aldrich) was added into 3 wells in each concentration group and incubated for 4 h at 37 °C in the dark place. After 150 μl DMSO (Sigma–Aldrich) was added, the absorbance at 490 nm was measured with a Multiskan GO microplate reader (Thermo Scientific).

2.4. EdU incorporation assay

The proliferation ability was measured by EdU incorporation assay. HCEP cells were cultured and treated with 1.25 g/l atropine as described above. At a 4 h interval, the culture medium of 3 wells in each group was replaced with 10% (v/v) FBS-DMEM medium supplemented with 50 nM EdU and incubated at 37 °C for 2 h. The cells were fixed with 4% (v/v) formaldehyde for 15 min and treated with 0.5% (v/v) Triton X-100 for 20 min at room temperature. After washed three times with PBS, the cells were incubated with 200 μl Apollo reaction cocktail (RiboBio, Guangzhou, China) for 30 min. Then the cells were stained with 200 μl of 10 μg/ml Hoechst 33342 for 20 min, and observed with a Ti-S fluorescent microscope (Nikon, Tokyo, Japan). In each group, six areas were randomly selected and the cell number, at least 300 cells, was counted respectively.

2.5. Acridine orange/ethidium bromide (AO/EB) double staining

The plasma membrane permeability was examined by AO/EB fluorescent double staining as described previously (Li et al., 2015). In brief, HCEP cells were cultured and treated with 0.15625–10 g/l atropine as described above. At a 2–4 h interval, the cells were harvested as described previously (Fan et al., 2011). The cells re-suspended in 0.1 ml of serum-free DMEM medium were stained 1 min with the addition of 4 μl of 100 μg/ml AO/EB solution (1:1) (Sigma–Aldrich) at room temperature. The stained cell suspension in each group was observed with a Ti-S fluorescent microscope. In each group, six areas were randomly selected and the cell number, at least 300 cells, was counted respectively. Apoptotic ratio (%) was calculated as "apoptotic cell number/total cell number × 100".

2.6. DNA electrophoresis

DNA fragmentation was detected by gel electrophoresis as described previously (Li et al., 2015). Briefly, HCEP cells were cultured in 25 cm^2 culture flasks, treated with 1.25–2.5 g/l atropine and harvested at a 4 h interval as described above. The genomic DNA was isolated with a Quick Tissue/Culture Cells Genomic DNA Extraction Kit (CWbiotech, Beijing, China). The DNA preparation from each group was loaded onto a 1% agarose gel and electrophoresed at 200 mA for 260 min. After stained with 0.5 mg/l EB solution for 10 min, the gel was observed with an EC3 Imaging System (UVP, LLC Upland, CA, USA).

2.7. Transmission electron microscopy (TEM)

The ultrastructure of HCEP cells was observed by TEM. HCEP cells were cultured in 25 cm^2 culture flasks, treated with 1.25 g/l atropine and harvested at a 4 h interval as described above. The cell pellet was fixed with 40 g/l glutaraldehyde in 0.1 M sucrose with 0.2 M sodium cacodylate buffer (pH 7.4) overnight at 4 °C. After washed with sodium cacodylate buffer, the cells were re-fixed with 10 g/l osmium tetroxide for 1.5 h. Following routine dehydration, epoxy resin embedding and ultrathin section, the specimens were stained with 20 g/l uranyl acetate–lead citrate and observed with an H700 transmission electron microscope (Hitachi, Tokyo, Japan).

2.8. ELISA test

The caspase activation was detected by ELISA using the active form of caspase antibody as described previously (Yu et al., 2014). In brief, HCEP cells were cultured in 24-well culture plates, treated with 1.25 g/l atropine and harvested at a 2 h interval as described above. Whole-cell protein extracts were obtained by lysing cells with 500 μl RIPA supplemented with PMSF (Biotime, Beijing, China). A high binding 96-well microtitre plate (Nunc) was coated with 100 μl of the protein extracts from each group overnight at 4 °C. After blocked with 5% non-fat milk (BD Bioscience, NJ, USA), the plate was incubated with 100 μl of rabbit anti-human IgG monoclonal antibody to caspase-2, -3 and -9 (active form) antibodies (Santa Cruz, CA, USA) (1:500) at 37 °C for 2 h, and 100 μl of HRP-conjugated goat anti-rabbit IgG monoclonal antibody (Santa Cruz; 1:2000) at 37 °C for 1 h, respectively. A colorimetric reaction was induced by 1% tetramethylbenzidine (TMB) substrate for 15 min at room temperature in the dark and stopped with 50 μl 0.5 M H_2SO_4. The 490 nm absorbance of each well was measured using a Multiskan GO microplate reader (Thermo Scientific).

2.9. Flow cytometry (FCM)

The cell cycle, phosphatidylserine (PS) externalization and mitochondrial transmembrane potential (MTP) were detected by FCM as described previously (Yu et al., 2014). In short, HCEP cells were cultured in 6-well culture plates, treated with 1.25 g/l atropine and harvested at a 4 h interval as described above. For cell cycle assay, 1 ml 70% alcohol was added to the cell pellet and mixed for overnight fixation at 4 °C. The cells were washed and resuspended in 500 μl propidium iodide (PI) (BD Biosciences, San Jose, CA, USA), and incubated in the dark for 30 min. For PS externalization assay, 5 μl FITC-labeled Annexin-V and 5 μl PI (BD Biosciences) were added into 500 μl of cell suspension, and incubated in the dark for 15 min. For MTP assay, 5 μl of 10 μg/ml JC-1 (Sigma–Aldrich) was added into 500 μl cell suspension, and incubated for 15 min in the dark. The stained HCEP cells were assayed with a FC500 MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA).

2.10. Western blotting

The protein expression was measured by western blotting as described previously (Li et al., 2012b). Briefly, HCEP cells were

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