·Basic Research·

Cytotoxic effects of betaxolol on healthy corneal endothelial cells both *in vitro* and *in vivo*

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Abstract

• AIM: To demonstrate the cytotoxic effect of betaxolol and its underlying mechanism on human corneal endothelial cells (HCE cells) *in vitro* and cat corneal endothelial cells (CCE cells) *in vivo*, providing experimental basis for safety anti-glaucoma drug usage in clinic of ophthalmology.

• METHODS: *In vivo* and *in vitro* experiments were conducted to explore whether and how betaxolol participates in corneal endothelial cell injury. The *in vitro* morphology, growth status, plasma membrane permeability, DNA fragmentation, and ultrastructure of HCE cells treated with 0.021875-0.28g/L betaxolol were examined by light microscope, 3-(4,5)-dimethylthiahiazo (-z -y1) -3,5 -di -phenytetrazoliumromide (MTT) assay, acridine orange (AO)/ethidium bromide (EB) double-fluorescent staining, DNA agarose gel electrophoresis, and transmission electron microscope (TEM). The *in vivo* density, morphology, and ultrastructure of CCE cells, corneal thickness, and eye pressure of cat eyes treated with 0.28g/L betaxolol were investigated by specular microscopy, applanation tonometer, alizarin red staining, scanning electron microscope (SEM), and TEM.

• RESULTS: Exposure to betaxolol at doses from 0.0875g/L to 2.8g/L induced morphological and ultrastructural changes of *in vitro* cultured HCE cells such as cytoplasmic vacuolation, cellular shrinkage, structural disorganization, chromatin condensation, and apoptotic body appearance. Simultaneously, betaxolol elevated plasma membrane permeability and induced DNA fragmentation of these cells in a dose-dependent manner in AO/EB staining. Furthermore, betaxolol at a dose of 2.8g/L also induced decrease of density of CCE cells *in vivo*, and non-hexagonal and shrunk apoptotic cells were also found in betaxolol-treated cat corneal endothelia.

• CONCLUSION: Betaxolol has significant cytotoxicity on HCE cells *in vitro* by inducing apoptosis of these cells, and induced apoptosis of CCE cells *in vivo* as well. The findings help provide new insight into the apoptosis-inducing effect of anti-glaucoma drugs in eye clinic.

• KEYWORDS: betaxolol; cytotoxicity; apoptosis; human corneal endothelial cells; cat corneal endothelial cells

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INTRODUCTION

Glaucoma is a common disease that causes blindness[1]. Betaxolol, a selective β receptor antagonist that reduces intraocular pressure (IOP) has been widely used in primary open-angle glaucoma (POAG) therapy[2]. However, prolonged usage of betaxolol often results in conjuctival hyperemia, decreased corneal sensitivity, and superficial punctata keratitis[3,4].

Human corneal endothelial cells (HCE cells), constituting the boundary between the corneal stroma and anterior chamber, play a vital role in maintaining corneal transparency and thickness [5]. In many species including humans, these cells retain little proliferating capacity throughout the normal lifespan and cell density continues to decrease throughout life [6]. Thus, damage to these cells will lead to endothelial decompensation, corneal edema, decreased corneal clarity and eventual loss of vision [7]. Therefore, assessing the potential toxicity of betaxolol on HCE cells is of great importance. As reported, betaxolol has cytotoxic effects on primary cultured HCE cells and bovine corneal endothelial cells by reducing their survival rate and increasing release of lactate dehydrogenase, respectively[8,9]. However, its cytotoxic effects on HCE cells are still unraveled, let alone the underlying mechanism.

The mechanisms of betaxolol-induced toxicity of HCE cells have been poorly investigated. The recently established untransfected HCE cell line makes it possible to evaluate the cytotoxicity of betaxolol on HCE cells and its underlying mechanisms *in vitro* [10]. In addition, cat corneal endothelial (CCE) cells provide a good *in vivo* model for assessing the cytotoxicity of betaxolol on mammalian corneal endothelial cells because CCE cells in adult cats have reduced proliferative ability just like those in humans[11].

In this study, the cytotoxicity of betaxolol on corneal endothelial cells and its underlying mechanisms were investigated with an *in vitro* model of HCE cells and an *in vivo* model of cat corneas.

MATERIALS AND METHODS

Materials Betaxolol powder (CAS#63659-18-7; 98% purity) was purchased from Stanford Chemicals (Irvine, CA, USA). Two-fold stock solutions and serial dilutions, from 2.8g/L to 0.021875g/L, were prepared in Dulbecco's modified Eagle medium: Ham's nutrient mixture F-12 (1:1) medium (DMEM/F12; Invitrogen, Carlsbad, CA, USA). One of untransfected HCE cell lines (utHCEC01) established previously in our laboratory were cultured in 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) containing DMEM/F12 medium (Invitrogen) and maintained at 37℃ in 25cm^2 culture flasks [10]. Betaxolol hydrochloride ophthalmic drops were purchased from Alcon Labratories Inc (Ft. Worth, TX, USA).

Animals An *in vivo* toxicity study was conducted on male domestic cats (*Felis domesticus*) (average body weight 2.0-2.5kg, age: 4 months) purchased from the Animal Center of Qingdao Chunghao Biotech company (Qingdao, China). Their care and usage for toxicity study were approved by Institutional Animal Care and Use Committee (IACUC) of the company. Ophthalmologic exams of the animals were performed and only the animals without any pre-existing corneal diseases were used in this study. The experimental procedures were carried out according to the American College of Toxicology Statement on the Use of Animals in Toxicology and approved by the Ethics Committee of Qingdao Chunghao Biotech company.

Experimental Design For *in vitro* experiments, cultured HCE cells at logarithmic phase were added to betaxolol solutions with concentrations of 2.8g/L, 1.4g/L, 0.7g/L, 0.35g/L, 0.175g/L, 0.0875g/L, 0.04375g/L, and 0.021875g/L respectively, and the HCE cell morphology and growth status, membrane integrity, DNA fragmentation, and ultrastructure were examined. Similarly cultured and maintained HCE cells without any addition of betaxolol were used as controls. For *in vivo* experiments, cat corneas were treated with 0.28g/L betaxolol topical drops, and cat corneal endothelial (CCE) cells and corneal thickness, together with CCE cell density, size, and hexagonality, were investigated.

Methods

Morphological observation of human corneal endothelial cells HCE cells were harvested from culture flasks by trypsin digestion and centrifugation as described previously, and seeded into a 24-well culture plate and cultured in the same medium as described above at 37℃ in a 5% CO_2 incubator [12]. Cultured HCE cells at logarithmic phase were added to betaxolol solutions with concentrations ranging from 2.8g/L to 0.021875g/L in 10% FBS-DMEM/F12 medium, and cultured at the same conditions as above. The morphology and growth status of the cells were monitored using an Eclipse TS100 inverted light microscope (Nikon, Tokyo, Japan) every 4h.

The 3 -(4,5) -dimethylthiahiazo (-z -y1) -3,5 -di -phenytetrazoliumromide assay HCE cells were seeded into a 96-well cell culture plate at a density of 1×10^4 cells per 200μL per well and cultured 48h prior to 3- (4, 5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide(MTT) assay. After the cells were treated 20h with betaxolol, the medium was replaced with an equal volume of fresh medium containing 1.1mmol/L MTT (Sigma-Aldrich) and incubated for 4h at 37℃ in the dark. The medium was discarded, and 150μL of dimethyl sulfoxide (Sigma-Aldrich) was added to dissolve the formazan produced. Cell viability was determined by a colorimetric comparison of the optical density values of the samples using a microplate reader (Multickan GO, Thermo Scientific) at an absorption wavelength of 590nm.

Plasma membrane permeability detection of human corneal endothelial cells Plasma membrane permeability was measured by Acridine Orange/Ethidium Bromide (AO/EB) double staining as described previously[13]. In brief, HCE cells were exposed to betaxolol solution at concentrations ranging from 2.8g/L to 0.021875g/L and cultured at the same conditions as above. The cells were harvested at 2 or 3h intervals by trypsin digestion for 30h post-treatment as described above. Cell pellets were re-suspended in 0.1mL serum-free DMEM/F12 medium containing 4μL of AO/EB staining solution (Sigma-Aldrich, St. Louis, MO, USA)(100mg/L AO:100mg/L EB=1:1). After staining about 1min at 25℃, 10μL of dyed cell suspension was dropped onto glass slides and observed using a Ti-S fluorescent microscope (Nikon). HCE cells with nuclei staining green are considered to have maintained plasma membrane integrity, while cells in which the nuclei stained orange have lost plasma membrane integrity. Apoptotic cells

can be distinguished from non-apoptotic cells on the basis of the absence or presence of nuclear condensation and fragmentation. The apoptotic rate of HCE cells was calculated according to the formula: "apoptotic rate (%)= apoptotic cells/(apoptotic cells+non-apoptotic cells)×100"[13].

DNA electrophoresis of human corneal endothelial cells DNA preparation and its electrophoresis were carried out as described previously [14]. HCE cells cultured in 25cm^2 flasks were added to betaxolol solutions with concentrations ranging from 0.175g/L to 2.8g/L. After HCE cells were harvested by the same method as described above and cellular DNA was isolated using a Quick Tissue/Culture Cells Genomic DNA Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). The DNA sample from each exposed cell culture was electrophoresed with 10g/L agarose gel (50V, 4h), and the gel was observed with an EC3 Imaging System (UVP, LLC, Upland, CA, USA) after stained with 0.5mg/L EB.

Ultrastructural examination of human corneal endothelial cells After treated with 0.175-2.8g/L betaxolol for 5, 20, and 30h, HCE cells were collected and fixed with 40g/L glutaraldehyde in 0.1mol/L sucrose with 0.2mol/L sodium cacodylate buffer (pH7.4) overnight at 4℃. After being washed with sodium cacodylate buffer and post-fixing with 10g/L osmium tetroxide for 1.5h, the fixed cells were dehydrated and embedded in epoxy resin. Ultrathin sections were stained with 20g/L uranyl acetate-lead citrate and observed by an H700 transmission electron microscope (TEM; Hitachi, Tokyo, Japan).

***In vivo* evaluation of betaxolol cytotoxicity on cat corneal endothelial cells** The *in vivo* cytotoxicity of betaxolol was investigated by using cat eyes and CCE cells. The 2 drops of 2.8g/L betaxolol hydrochloride (betaxolol hydrochloride ophthalmic drops, Alcon Labratories Inc., Fort Worth, TX, USA) was used at 3 times a day for 3d in right eyes of 4 cats. The left eyes of them were untreated and served as controls. The density, hexagonality, and size of CCE cells, and central corneal thickness and IOP were monitored by using an SP-3000P specular microscope (Topcon, Tokyo, Japan) every 5d. Forty-five days after exposure to betaxolol, the corneas were taken out. CCE from the left and right eyes of each cat were stained with 10g/L alizarin red staining and examined using JSM-840 scanning electron microscope (SEM; JEOL, Tokyo, Japan)[15].

Statistical Analysis Experiments in this study were repeated 4-6 times. Data were presented as mean ±SEM in quadruplicates and analyzed for statistical significance with ANOVA single factor. Changes were considered statistically significant if $P<0.05$.

RESULTS

Morphological Changes of Human Corneal Endothelial Cells Effects of betaxolol exposure on the HCE cell morphology *in vitro* is shown in Figure 1. HCE cells exposed to 0.04375-2.8g/L betaxolol exhibited cytoplasmic vacuolation, cell shrinkage, detachment from culture matrix, and cell death. The morphological changes were dose- and time-dependent. Betaxolol exposure at concentrations below dose of 0.021875g/L showed no difference from those of controls. All these imply that betaxolol above dose of 0.04375g/L has significant cytotoxicity on HCE cells in *vitro*.

Viability Changes of Human Corneal Endothelial Cells HCE cells treated with betaxolol from concentration of 0.0875g/L to 2.8g/L exhibited obvious viability decrease ($P<0.05$) detected by MTT assay, while those treated with betaxolol below the concentration of 0.0875g/L showed no obvious viability changes (Figure 2).

Plasma Membrane Permeability Changes of Human Corneal Endothelial Cells AO/EB staining showed that plasma membrane permeability of HCE cells treated with 0.175-2.8g/L betaxolol was elevated, while that of the cells treated with 0.021875-0.0875g/L betaxolol showed no significant difference from that of control cells (Figure 3).

DNA Fragmentation of Human Corneal Endothelial Cells Agarose gel electrophoresis showed that the genomic DNAs from HCE cells exposed to betaxolol exhibited DNA ladders, while no DNA ladders were found in control HCE cells (Figure 4). These results suggest that DNA fragmentation occurred after HCE cells were exposed to betaxolol.

Ultrastructurcal Changes in Human Corneal Endothelial Cells Results of TEM observation of HCE cells exposed for 15h to 0.7g/L betaxolol are shown in Figure 5. Some of the cells exhibited typical ultrastructural characteristics of early-stage apoptosis such as cytoplasmic vacuolation, structural disorganization, and chromatin condensation (Figure 5A). Some cells exhibited ultrastructural changes consistent with late-stage apoptosis, including advanced structural disorganization, advanced chromatin condensation with inner-nuclear marginal localization, disaggregation of cell and nucleus, and presence of many apoptotic bodies (Figures 5B, and 5C). Control HCE cells preserved normal ultrastructure after culture for 15h (Figure 4D). These results suggest that betaxolol (at exposure concentrations of 0.7g/L) does have an apoptosis-inducing effect on HCE cells *in vitro*.

Effect of Betaxolol on Cat Corneal Endothelial Cells *in vivo* *In vivo* specular microscopy showed that the average cell density of CCE cells from cat corneas exposed to 2.8g/L betaxolol decreased and average cell size increased with time, compared with those from normal control eyes ($P<$

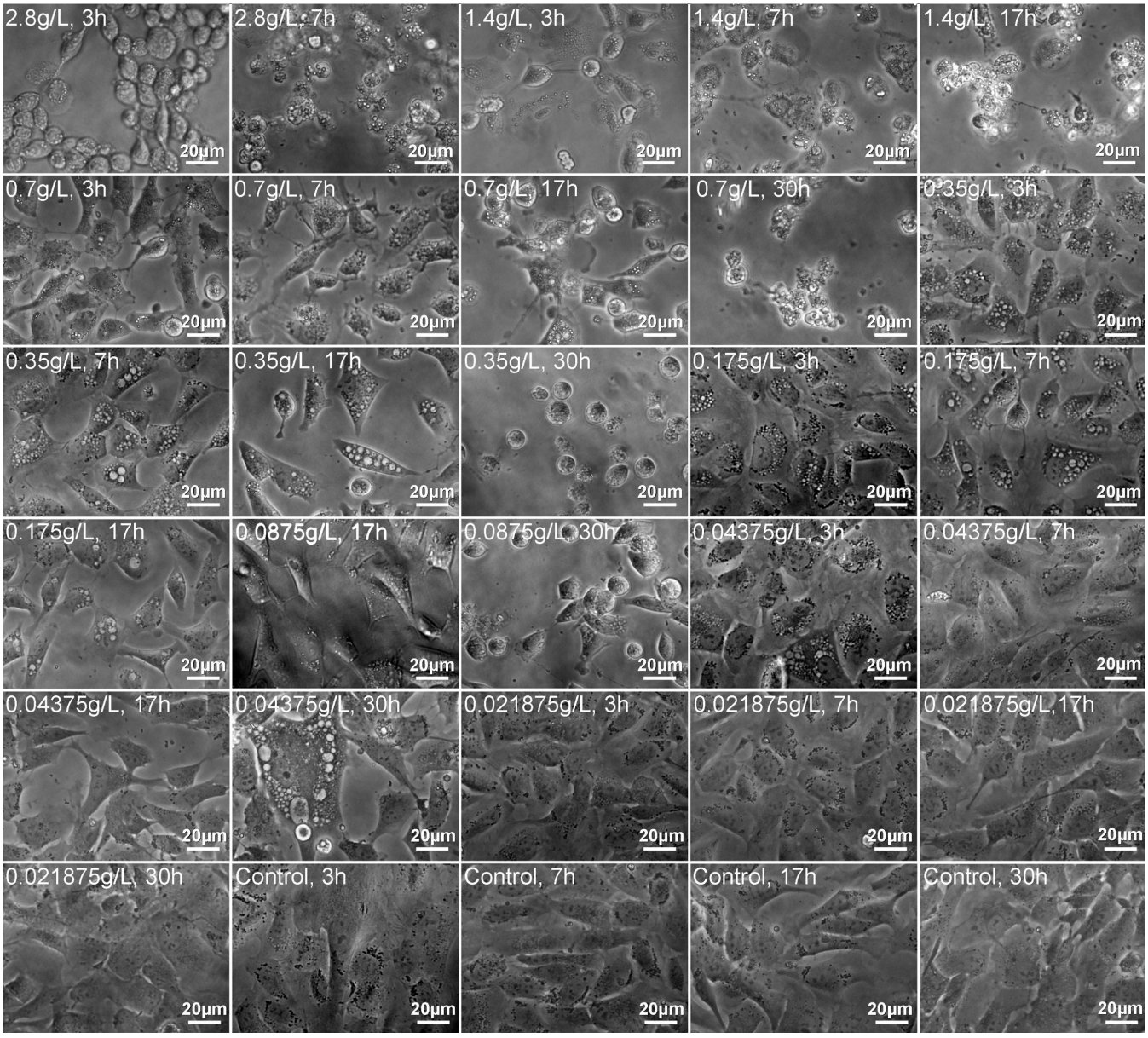

Figure 1 Betaxolol induces light-microscopic morphological changes of HCE cells Cultured HCE cells were treated with or without various concentrations of betaxolol (0.021875-0.28g/L) for different time, and observed by inverted light microscopy. The dosage and time of betaxolol treatment are shown in the top-left of each photograph.

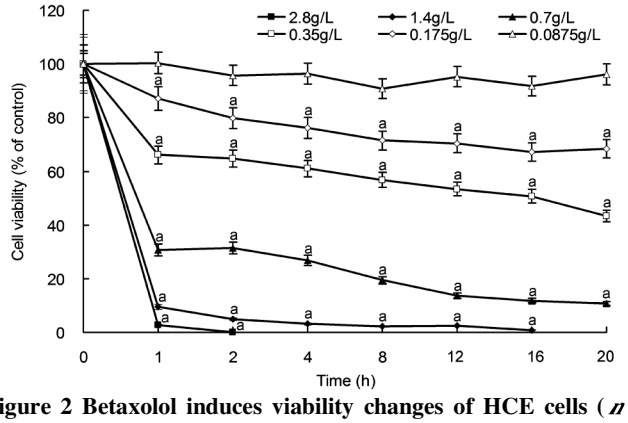

Figure 2 Betaxolol induces viability changes of HCE cells (n=6) Betaxolol at the concentration above 0.0875g/L showed viability decrease detected by MTT assay. [a]P<0.05.

0.05) (Figure 6A, 6B). The corneal thickness and IOP of betaxolol-treated eyes showed no significant difference compared with the normal control eyes (Figure 6C, 6D).

Morphology Changes of Cat Corneal Endothelial Cells *in vivo* The CCE cells of corneas taken out from cat eyes were examined by alizarin red staining and SEM. Results showed that non-hexagonal, shrunk and apoptotic-like CCE cells appeared in the corneal endothelia of betaxolol-treated cat eyes in alizarin red staining assay (Figure 7A, and 7B), and this was further verified in SEM detection (Figure 7C, 7D). These results suggest that betaxolol also may have induced apoptosis in CCE cells *in vivo*.

Ultrastructural Changes of Cat Corneal Endothelial Cells TEM observations showed that the CCE cells in betaxolol-treated corneas exhibited apoptotic-like ultra structural characteristics, *i.e.* cellular shrinkage and cytoplasmic vacuolation compared with control (Figure 8).

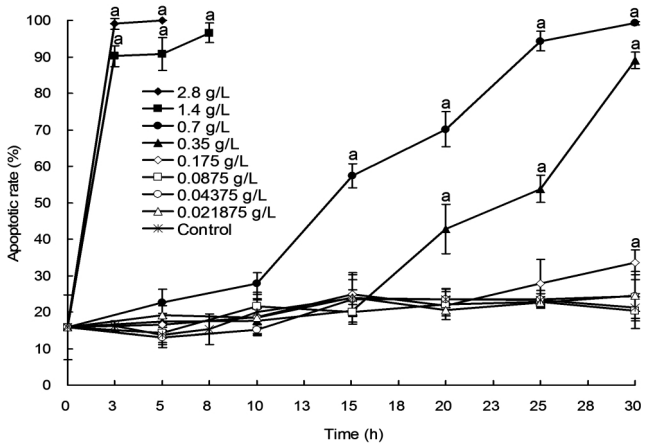

Figure 3 Betaxolol induces plasma membrane permeability changes of HCE cells (n=6) Cultured HCE cells were treated with or without various concentrations of betaxolol (0.021875-0.28g/L) for 30h, and observed every 2.5h or 5h by fluorescent microscopy with acridine orange/ethidium bromide (AO/EB) double staining. [a] P<0.05.

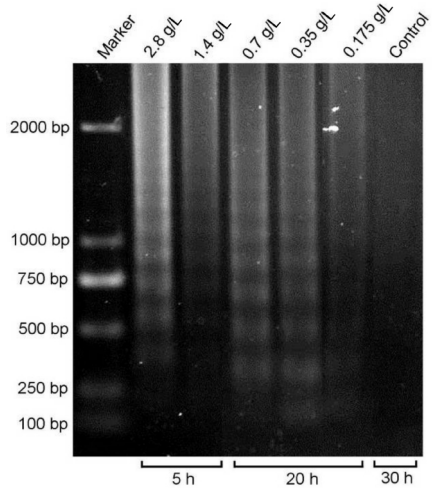

Figure 4 Effect of betaxolol on the DNA fragmentation of HCE cells DNA fragmentation in HCE cells treated with or without various concentrations of betaxolol (0.175-2.8g/L) for 5, 20 or 30h was detected by 10g/L agarose electrophoresis, respectively. Marker: D2000 standard molecular weight marker; bp: Base pair.

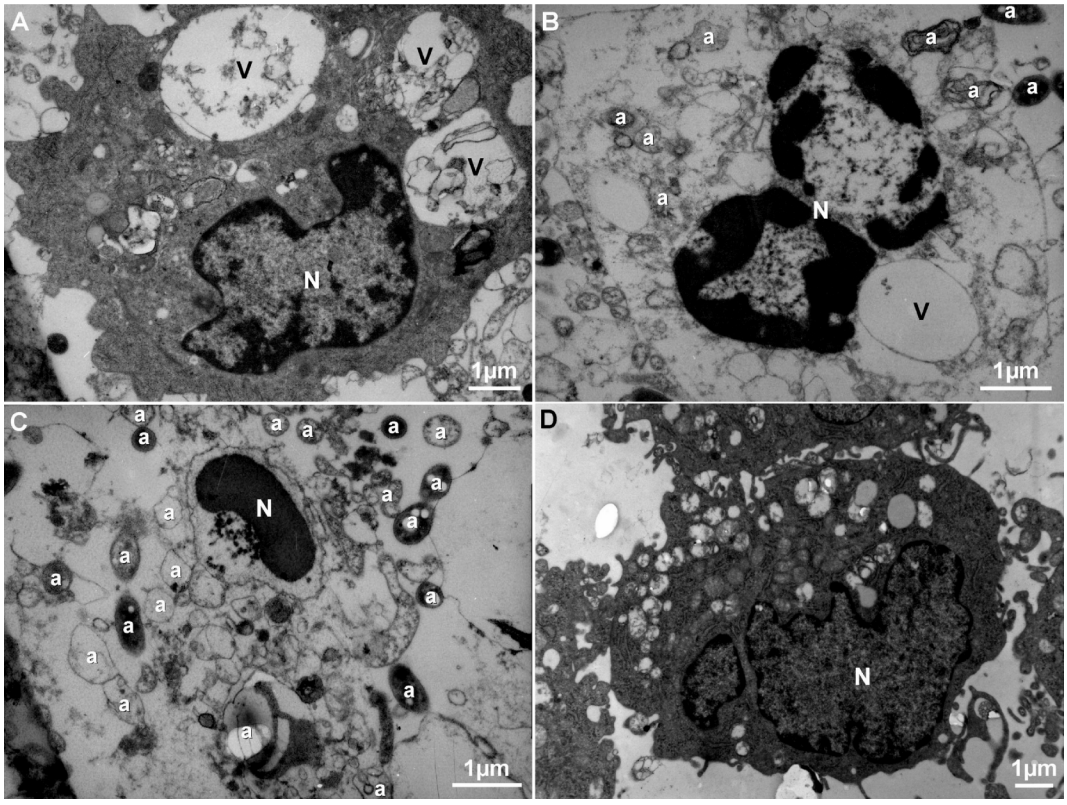

Figure 5 Effect of betaxolol on the ultrastructure of HCE cells *in vitro* The ultrastructure of HCE cells from 0.7g/L betaxolol treated group (A-C) and non-treated control group (D) were analyzed by TEM. N: nucleus; V: vacuole; a: apoptotic body.

DISCUSSION

Betaxolol is a selective β receptor antagonist which has been widely used in POAG therapy to reduces IOP [2]. But prolonged usage of betaxolol could cause many problems, such as apoptosis on corneal endothelial cells, conjuctival hyperemia, decreased corneal sensitivity, and superficial punctata keratitis[3,4]. However, the apoptotic effect and exact mechanism involved in cytotoxicity of betaxolol not well established in HCE cells.

By the utilization of an HCE cells *in vitro* and cats' corneas *in vivo* we evaluated the cytotoxicity of betaxolol on corneal endothelial cells and its mechanisms of toxicity. Light microscopic observations on cellular morphology showed that HCE cells, exposed to betaxolol at concentrations above 0.0875g/L (1/32 of the routine clinical dosage of 2.8g/L) were associated qualitative morphological changes including cytoplasmic vacuolation, cellular shrinkage, detachment, and death, which means apoptosis occurred on cells [16,17]. Further

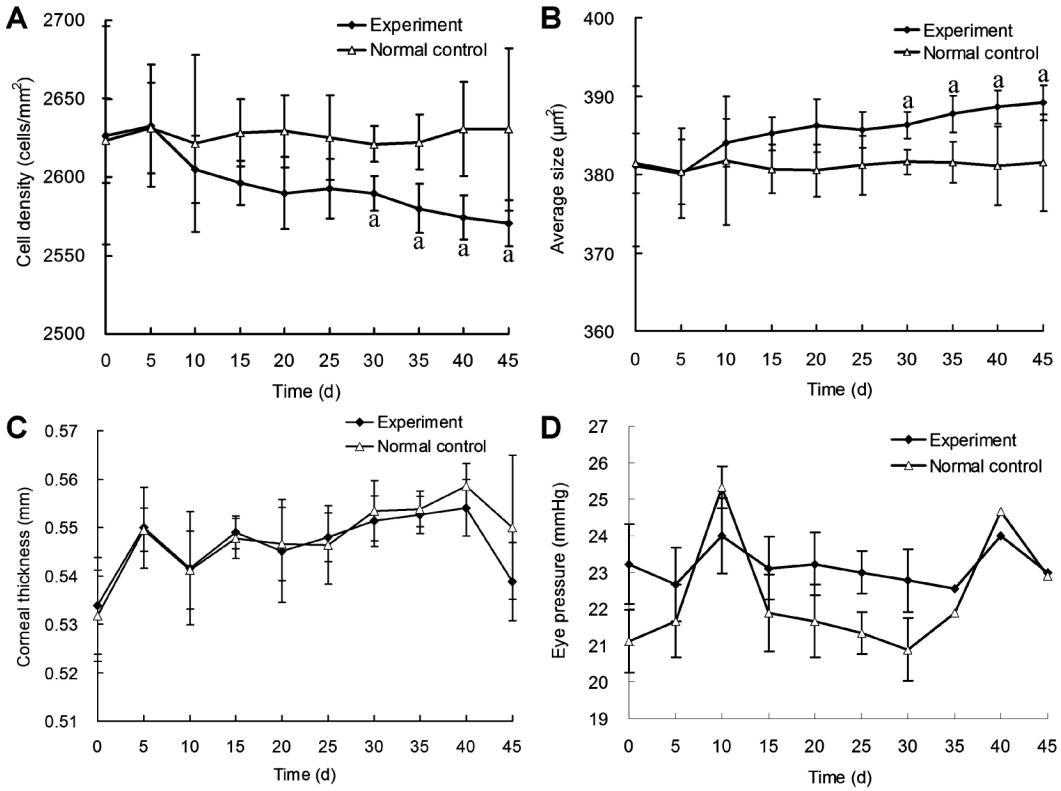

Figure 6 Effect of betaxolol on cat corneas *in vivo* (n=4) The right eye cornea of each cat was treated with 2.8g/L betaxolol and analyzed by specular microscopy, while the left eye cornea of each cat was used as control. A: CCE cell density; B: Average size; C: Corneal thickness; D: IOP. ^aP<0.05.

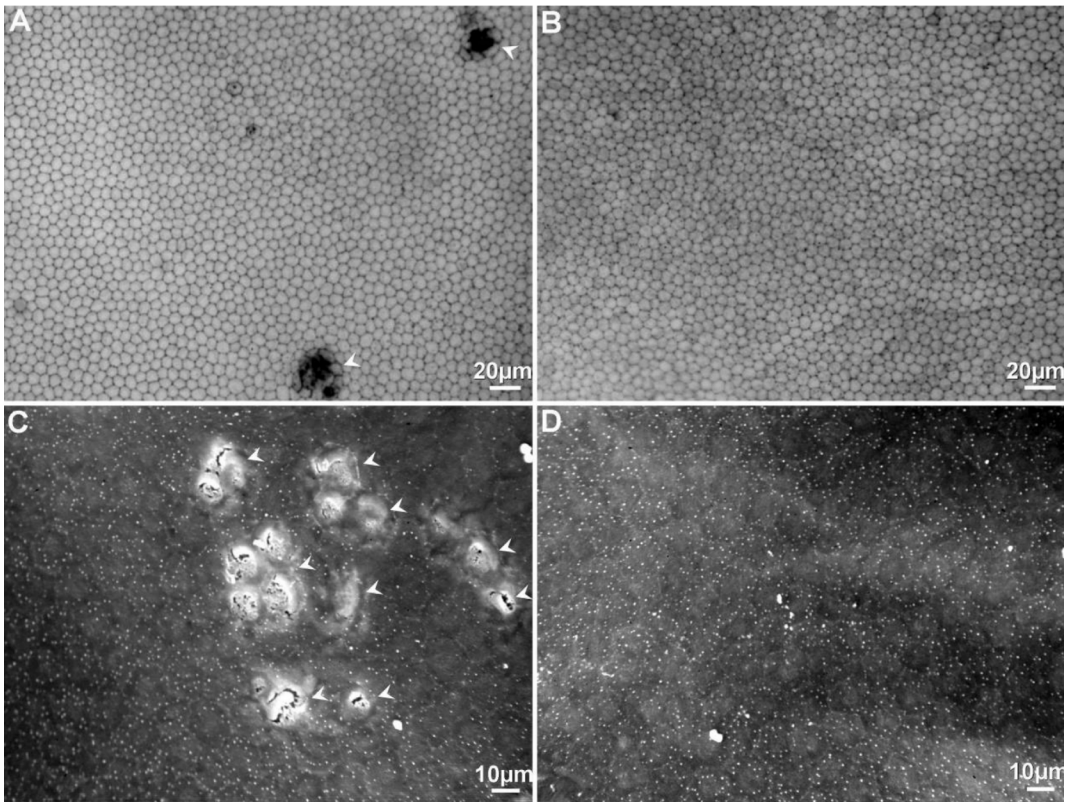

Figure 7 Effect of betaxolol on the histological structure of CCE cells *in vivo* The corneas treated with betaxolol (A and C) or without betaxolol (2.8g/L) (B and D) were taken out at day 45, respectively. The morphology of CCE cells were analyzed by alizarin red staining (A and B) and SEM (C and D). Arrow head: apoptotic cell(s).

result of our study in MTT assay showed that HCE cells which were treated with betaxolol above the concentration of 0.0875g/L exhibited obvious viability decrease, which implied that the viability of the HCE cells were influenced also.

The increasing of plasma membrane permeability has been

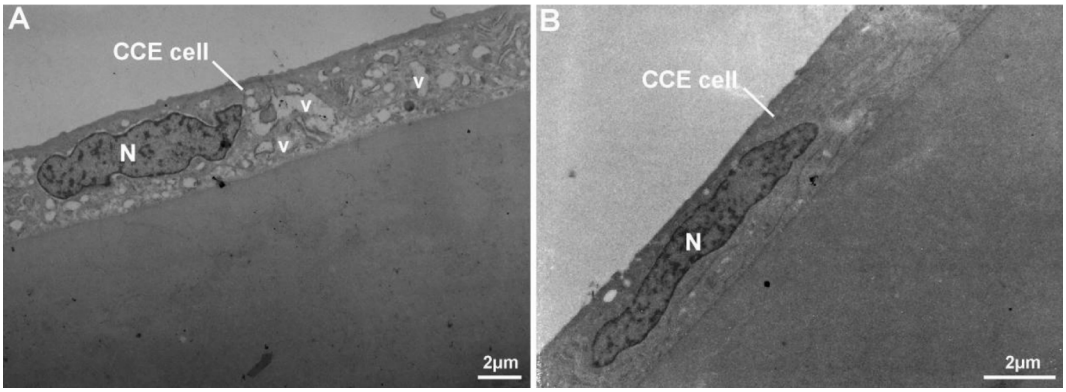

Figure 8 The corneas treated with 2.8g/L betaxolol (A) compared with control (B) were taken out at day 45 The ultrastructural changes of CCE cells were observed by TEM showed that cellular shrinkage and cytoplasmic vacuolation occurred in the betaxolol-treated eyes. N: nucleus; v: Vacuole.

reported as a typical feature of apoptotic cells, so we observed whether plasma membrane permeability of HCE cells exposed to betaxolol has changed [13,18]. The double-fluorescent staining of AO/EB showed that plasma membrane permeability of betaxolol-exposed HCE cells increased after betaxolol-exposed. However, another proves of DNA fragmentation in betaxolol-exposed HCE cells was examined by agarose gel electrophoresis. The DNA fragmentation is one of most remarkable features of apoptotic cells [14,19]. Our result showed that classical DNA ladders were present after electrophoresis, indicating that DNA fragmentation occurred in betaxolol-treated HCE cells. Both results strongly suggest that betaxolol might have an apoptosis-inducing effect on HCE cells.

To evaluate the ultrastructural features of betaxolol-exposed HCE cells, TEM found that structural disorganization, chromatin condensation and its inner-nuclear marginal location, disaggregation of cell and nucleus, and presence of many apoptotic bodies were observed in betaxolol-exposed HCE cells, all of which are ultrastructural features typical of apoptotic cells[20,21].

Thus, the qualitative TEM and light microscopic morphological changes, plasma membrane permeability elevation, DNA fragmentation results suggest that betaxolol can induce cytotoxicity in HCE cells *in vitro*, an effect which may be mediated by induction of apoptosis.

An *in vivo* model (cat eyes and CCE cells) was also utilized to investigate the cytotoxicity of betaxolol, according to the evidence of cytotoxicity of most clinically used agents obtained in a cell culture system needs to be validated using *in vivo* models [22]. After 45d exposure of betaxolol, the corneas were taken out. The IOP of betaxolol-treated eyes showed no significant difference to that of normal control eyes, which might be explained by the less effect of anti-glaucoma drugs on non-glaucomatous eyes. The results of alizarin-red staining and SEM showed that non-hexagonal and apoptotic-like CCE cells was observed in betaxolol-exposed eyes, which implies that betaxolol can induce cytotoxicity in CCE cells *in vivo*. However, the result of TEM about ultrastructural changes of CCE cells once again showed that cellular shrinkage and cytoplasmic vacuolation occurred in the betaxolol-treated eyes.

In conclusion, the results on HCE cells *in vitro* and on CCE cells *in vivo* together strongly indicate that betaxolol has cytotoxic effect on corneal endothelial cells by inducing apoptotic cell death. It should be noted that the usage of this anti-glaucoma drug in eye clinic may be a potential risk factor for corneal endothelial injury. Further studies should be exploited for overcoming its cytotoxicity in eye clinic.

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