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LABORATORY STUDY

Comparison of fluoroquinolones: cytotoxicity on human corneal epithelial cells

T-H Tsai, W-L Chen and F-R Hu

Abstract

Purpose **To compare the cytotoxicity of different fluoroquinolones (FQs) towards human corneal epithelial cells (HCECs).**
Methods **HCECs were incubated with FQs (norfloxacin, ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin, and gatifloxacin), both as commercial ophthalmic formulations and as unpreserved solutions. Cells incubated in different formulations of gentamicin, cefazolin, and benzalkonium chloride (BAC) were also compared. A cell viability assay, using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, was used to evaluate the drug effects on cell viability after five incubation times (30 min, 1 h, 4 h, 8 h, and 24 h). Transepithelial electrical resistance (TEER) was measured with a voltohmmeter to help understand changes in paracellular permeability at five time points (4 h, 8 h, 12 h, 24 h, and 48 h). Cell morphology was observed with an inverted fluorescence microscope, with multiple stage position and in time-lapse mode.**
Results **The preserving solutions and BAC at concentrations above 0.005% significantly decreased cell viability, when assayed by MTS. Increased paracellular permeability and decreased membrane integrity were also observed by TEER measurements and inverted fluorescence microscopy. Ofloxacin and levofloxacin were both free of preservatives and showed the least cytotoxicity towards HCECs in commercial FQ eye drops.**
Conclusions **The cytotoxicity observed with FQ eye drops seems to be caused mainly by the preservative, which induced a significant decrease in membrane integrity and increased paracellular permeability. We found the new generation of FQs (moxifloxacin and gatifloxacin) no less cytotoxic towards HCECs than the old generation ones.**

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Introduction

Bacterial keratitis is a common ocular infection and a leading cause of ocular morbidity and blindness worldwide. However, with appropriate antibiotics selected on the basis of diagnostic corneal smears and cultures, around 72% of infections could be successfully treated topically.[1] Fluoroquinolones (FQs) were derived from the non-fluorinated drug nalidixic acid, which was developed during the early 1960 s. FQs are potent antimicrobial agents with a broad antibacterial spectrum and are suitable for monotherapy. They act rapidly by inhibiting bacterial DNA gyrase and topoisomerase IV, which are selective for bacterial cells.[2] With different modifications to the basic molecular '4-quinolone' skeleton, several generations of FQs have been developed.[3,4] The hallmark of fourth-generation FQs is the 8-methoxy group (-OCH_3), which improves activity against Gram-positive pathogens, whereas retaining the potency of the third-generation agents against Gram-negative pathogens.[5] FQs are nowadays widely used in clinical practice to treat ocular infections.

In treating bacterial keratitis, both the drug efficacy and cytotoxicity should be considered when choosing antibiotics. Fourth-generation FQs (moxifloxacin and gatifloxacin) were introduced to ophthalmology to counteract the emerging resistance to third-generation ones

Department of Ophthalmology, National Taiwan University Hospital, Taipei, Taiwan

Correspondence: F-R Hu, Department of Ophthalmology, National Taiwan University Hospital, Medical College, National Taiwan University, No. 7, Chung-Shan South Road, Taipei 100, Taiwan.
Tel: 886-2-23123456 ext. 65189,
Fax: 886-2-23934420,
E-mail: fungronghu@ntu.edu.tw

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(ciprofloxacin, ofloxacin, and levofloxacin).[6–8] The successful treatment of mycobacterial keratitis with fourth-generation FQs has also been reported.[9–11] However, bacterial keratitis resistant to fourth-generation FQs has been found[12] and the prophylactic usage of moxifloxacin and gatifloxacin did not significantly lower the endophthalmitis rate after uncomplicated cataract surgery either.[13] Although the superiority of clinical efficacy between different FQs is still under evaluation,[14–20] controversial results exist that compare the toxicity of FQs towards the corneal epithelial cells.[19,21–23] The ocular and systemic safety profile of newer FQs is generally considered comparable with those of other FQ antimicrobial agents, with a low recognized risk of quinolone-related toxicity.[24–27] However, Walter *et al*[28] reported two cases of severe sterile corneal ulcers after the topical application of moxifloxacin, and the drug was suspected of interfering with corneal healing.

Several factors may contribute to the cytotoxicity of FQ eye drops. Besides the toxicity from the antibiotics itself, other components of the formulation including osmolarity, pH, and the presence of the preservatives benzalkonium chloride (BAC) may all contribute. The purpose of our study is to compare the cytotoxicity between different FQs and other commonly used antibiotics, including commercial gentamicin eye drops (0.3%), unpreserved fortified antibiotics (1.4% gentamicin, and 5% cefazolin) and BAC (0.001, 0.005, and 0.01%). The results could serve as a reference for the clinical application of FQs, as well as help clarify the source of cytotoxicity within these drugs.

Materials and methods

Test materials

We used both the raw materials (standard powders) and commercially available ophthalmic solutions of FQs as testing materials. The standard powders were sourced as follows: norfloxacin (Sigma-Aldrich Inc., Milwaukee, WI, USA), ciprofloxacin (Bayer AG, Leverkusen, Germany), ofloxacin (Daiichi Pharmaceutical Co., Tokyo, Japan), levofloxacin (Daiichi Pharmaceutical Co.), moxifloxacin (Bayer AG), and gatifloxacin (Kyorin Pharmaceutical Co., Tokyo, Japan). Ophthalmic solutions of the following were used in this study: norfloxacin (0.3%, Baccidal; Kyorin Pharmaceutical Co.), ciprofloxacin (0.3%, Ciloxan; Alcon Laboratories Inc., Puurs, Belgium), ofloxacin (0.3%, Oflovid; Santen Pharmaceutical Co., Osaka, Japan), levofloxacin (0.5%, Cravit; Santen Pharmaceutical Co.), moxifloxacin (0.5%, Vigamox; Alcon Laboratories Inc., Fort Worth, TX, USA), and gatifloxacin (0.3%, Zymar; Allergan Inc., Irvine, CA, USA). Also tested and compared against the FQs were gentamicin ophthalmic solution (0.3%, Garamycin; Schering-Plough, Brussels, Belgium), fortified antibiotic solution prepared from parental gentamicin (U-Gencin 40 mg/ml, U-Liang Pharmaceutical Co., Jhongli City, Taiwan), and cefazolin (Cefamezin 1.0 g/vial; Taiwan Biotech Co., Taoyuan, Taiwan) and BAC (Sigma-Aldrich Inc.).

Preparation of test solutions

The standard powders of norfloxacin, ciprofloxacin, ofloxacin, levofloxacin, and gatifloxacin were dissolved in distilled water to a stock concentration of 3% (w/v). A 1.8% moxifloxacin solution in distilled water was prepared. FQ stock solutions were diluted with medium (pH ~7.3) to 0.3% (market concentration) immediately before cell treatments. The pH was adjusted by 2N HCl titration to 6.5–7.0, except for norfloxacin (5.0–5.5) and ciprofloxacin (4.5–4.8). Preservative-free parental gentamicin was diluted to 1.4% with distilled water. Cefazolin powder was dissolved in distilled water and diluted to make a 5% solution (pH ~7) with distilled water before use. A stock solution of 0.2% BAC (pH 6.9) prepared in distilled water was further diluted to 0.01, 0.005, and 0.001% with distilled water and 0.0005% with culture medium before cell treatments. The antibiotics prepared herein did not contain preservatives or pharmaceutical excipients. Although not proven, the observed effects may mostly be attributed to the test solutions *per se*.

The ophthalmic solutions of gatifloxacin (Zymar) (pH 6.0) and gentamicin (Garamycin) (pH 7.0) contained 0.005% BAC. Ciprofloxacin solution (Ciloxan) (pH 4.5) contained 0.006% BAC. Norfloxacin (Baccidal) (pH 5.0–5.6), ofloxacin (Oflovid) (pH 6.0–7.0), levofloxacin (Cravit) (pH 6.2–6.8), and moxifloxacin (Vigamox) (pH 6.8) were BAC-free preparations. The osmolarities were all within 0.85–1.15 times that of normal saline. In transepithelial electrical resistance (TEER) measurements, we diluted these commercial drugs to 1/10 concentration with culture medium.

The medium used for test solutions preparation did not contain serum or antibiotics.

Cell culture

A human corneal epithelial cell line (HCECs) (ATCC CRL, 11515) was used in this study. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.125 μg/ml amphotericin B at 37°C in a moist atmosphere with 5% CO_2. The culture medium was changed every 2 days.

MTS assay

For viability studies, cells were seeded in 96-well plates (2×10^4 cells/well), incubated in DMEM and supplemented as described above for 24 h. Once a confluent cell layer was obtained, the culture medium was withdrawn and the cells were then incubated for the further 18 h with fresh medium without serum and antibiotics supplement. Afterwards, the media were removed and 100 μl of the test materials were added in various concentrations (market ophthalmic solution of FQs and gentamicin; preservative-free fortified antibiotics of 1.4% gentamicin and 5% cefazolin; 0.3% solutions of FQ powders and BAC solutions of 0.01, 0.005, and 0.001%). The MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) was performed at five incubation time points (30 min, 1 h, 4 h, 8 h, and 24 h). The test materials were removed and 20 μl of MTS reagent along with 100 μl of culture medium (without serum and antibiotics) were added to each well. HCECs were further incubated at 37°C for 3 h. Absorbance measurements were read at 490 nm using a fluorescence/absorbance 96-well plate reader afterwards. Negative control groups consisted of cells in media (without serum and antibiotics) without the test materials added (pH = 7.3). Groups of media, pH adjusted to 5.0 (without serum, antibiotics or test materials added), were processed identically and incubated alongside the treated groups. Three independent experiments ($n = 4$ per group) were done. Cell viability was expressed as a percentage of the negative control group.

To distinguish the toxicity from water and ensure the adequacy of nutrient for cell growth, we compared HCECs cultured in media without test materials (negative control) and those in distilled water (vehicle control). MTS assay were performed at 1 h, 4 h, 8 h, 12 h, 16 h, 20 h, and 24 h between these two groups.

Transepithelial electrical resistance

Transepithelial electrical resistance was measured with a voltohmmeter (Millicell-ERS, Millipore, Billerica, MA, USA) to help monitor changes in paracellular permeability. Cells (4.55×10^5 cells/insert) were seeded on transwell polycarbonate inserts (12-well format, 12 mm insert diameter) with a mean pore size of 0.4 μm (Transwell, Corning, Lowell, MA, USA) after being coated with fibronectin and serum-free DMEM (1 : 10). After 3 days, the cells reached confluency (raw resistance above 140 $\Omega\,cm^2$) and the medium was substituted with serum- and antibiotics-free medium for further culture for 24 h. When the raw resistance reached 200 $\Omega\,cm^2$, the medium was replaced with 2 ml (0.5 ml within insert and 1.5 ml outside the insert each well) of 0.0005% BAC (1/10 of the concentration present in market ophthalmic solutions), six commercial FQ and gentamicin drops at 1/10 of market concentration, for better observing the changes of paracelluar permeability. Cells grown in culture medium only (without serum and antibiotics) served as control group (pH = 7.3). Cells in media pH adjusted to 5.0 without any supplement were processed alongside the treated groups. Blanks (inserts without cells, containing media only) were used to determine background values (raw resistance around 110 $\Omega\,cm^2$). TEER was measured at 4 h, 8 h, 12 h, 24 h and 48 h. Three independent experiments ($n = 3$ per group per experiment) were done. The background TEER of blank transwell filters was subtracted from the TEER of the cell monolayer. TEER was calculated from the measured resistance and normalized by the area of the monolayer (ohms per cm^2). Values were expressed as percentage of the control groups at each time point.

Inverted fluorescence microscopy

Cell morphology was observed and recorded by inverted fluorescence microscopy (Axiovert 200 M, Zeiss, Thornwood, NY, USA). Cells were seeded in 24-well plate (8×10^4/well) incubated in DMEM, supplemented as described above for 24 h. The medium was withdrawn after the cells were confluent, and serum- and antibiotics-free medium was added for further incubation. After 18 h, the medium was removed and preservative-free 0.3% FQ solutions were added ($n = 2$ for each group). Multiple stage position, time-lapse microscopy was used to record the cell morphology during drug treatments. Images were taken every 10 min over a 16 h period.

Statistical analysis

Values were expressed as the mean percentages of control values ± SD from three independent experiments, each in four or three replicates ($n = 12$ in MTS assay and $n = 9$ in TEER). The *t*-tests were used to compare test group with the control group or with another test group. If normality or the equal variance test was not satisfied, the Mann–Whitney rank-sum test was applied. A P-value < 0.05 was considered as statistically significant. All statistical data were analysed between different groups using Kruskal–Wallis one-way analysis of variance on ranks and by pairwise multiple comparison (Dunn's method) (Sigmastat 2.03 SPSS Inc., Chicago, IL, USA). Bonferroni-adjusted P-value was used in multiple groups comparison, a P-value $< 0.05/n$ (where n is the number of groups) was considered as statistically significant.

Table 1 MTS assay (% of control): HCECs incubated with commercial fluoroquinolone and gentamicin solutions, parental fortified gentamicin 1.4% and cefazolin 5%, benzalkonium chloride (0.01, 0.005, 0.001%) and pH = 5 medium compared with untreated cells (negative control)

	30 min (Mean ± SD) (P-*value*)	*1 h (Mean ± SD)* (P-*value*)	*4 h (Mean ± SD)* (P-*value*)	*8 h (Mean ± SD)* (P-*value*)
Negative control	100.0 ± 1.81	100.0 ± 2.05	100.0 ± 4.69	100.0 ± 1.71
0.3% Baccidal	98.3 ± 1.78 ($P = 0.120$)	96.5 ± 10.90 ($P = 0.49$)	51.0 ± 4.58 ($P < 0.001$)*	37.4 ± 4.11 ($P < 0.001$)*
0.3% Ciloxan	39.4 ± 5.93 ($P < 0.001$)*	39.6 ± 3.85 ($P < 0.001$)*	39.1 ± 1.28 ($P < 0.001$)*	38.4 ± 3.35 ($P < 0.001$)*
0.3% Oflovid	98.7 ± 4.91 ($P = 0.486$)	100.8 ± 4.67 ($P = 0.574$)	65.0 ± 1.00 ($P = 0.057$)	55.3 ± 18.80 ($P < 0.001$)*
0.5% Cravit	95.1 ± 8.37 ($P = 0.13$)	96.8 ± 8.96 ($P = 0.213$)	54.6 ± 4.69 ($P = 0.057$)	49.0 ± 12.40 ($P < 0.001$)*
0.5% Vigamox	79.7 ± 16.55 ($P = 0.01$)#	67.7 ± 17.60 ($P < 0.001$)*	44.2 ± 8.89 ($P < 0.001$)*	38.4 ± 4.05 ($P < 0.001$)*
0.3% Zymar	49.5 ± 16.05 ($P < 0.001$)*	40.7 ± 3.07 ($P < 0.001$)*	40.6 ± 4.76 ($P < 0.001$)*	38.8 ± 3.14 ($P < 0.001$)*
0.3% Garamycin	45.1 ± 10.66 ($P < 0.001$)*	40.7 ± 5.72 ($P < 0.001$)*	40.1 ± 1.30 ($P < 0.001$)*	37.5 ± 3.57 ($P < 0.001$)*
1.4% Ugencin	90.6 ± 2.30 ($P = 0.002$)#	86.0 ± 5.56 ($P = 0.009$)#	86.6 ± 1.06 ($P = 0.057$)	85.6 ± 0.90 ($P < 0.001$)*
5% Cefamezin	97.5 ± 3.43 ($P = 0.108$)	100.0 ± 1.00 ($P = 1.00$)	88.0 ± 1.97 ($P = 0.057$)	81.4 ± 6.34 ($P = 0.007$)#
0.01% BAC	45.9 ± 9.09 ($P < 0.001$)*	41.1 ± 8.02 ($P < 0.001$)*	42.9 ± 1.84 ($P < 0.001$)*	37.7 ± 1.49 ($P < 0.001$)*
0.005% BAC	64.4 ± 16.60 ($P < 0.001$)*	45.2 ± 14.20 ($P < 0.001$)*	48.3 ± 2.37 ($P < 0.001$)*	39.6 ± 7.48 ($P < 0.001$)*
0.001% BAC	98.1 ± 5.12 ($P = 0.142$)	97.8 ± 4.17 ($P = 0.105$)	79.4 ± 6.68 ($P = 0.005$)#	48.3 ± 13.20 ($P < 0.001$)*
pH = 5 medium	95.9 ± 7.18 ($P = 0.662$)	98.6 ± 5.47 ($P = 0.378$)	97.6 ± 0.99 ($P = 1.00$)	97.8 ± 12.30 ($P = 0.589$)

SD, standard deviation.
Mann–Whitney rank-sum test compared with negative control. *$P < 0.001$, #$P < 0.05$.
Negative control: HCECs in pH = 7.3 fresh medium without serum or antibiotics supplements.

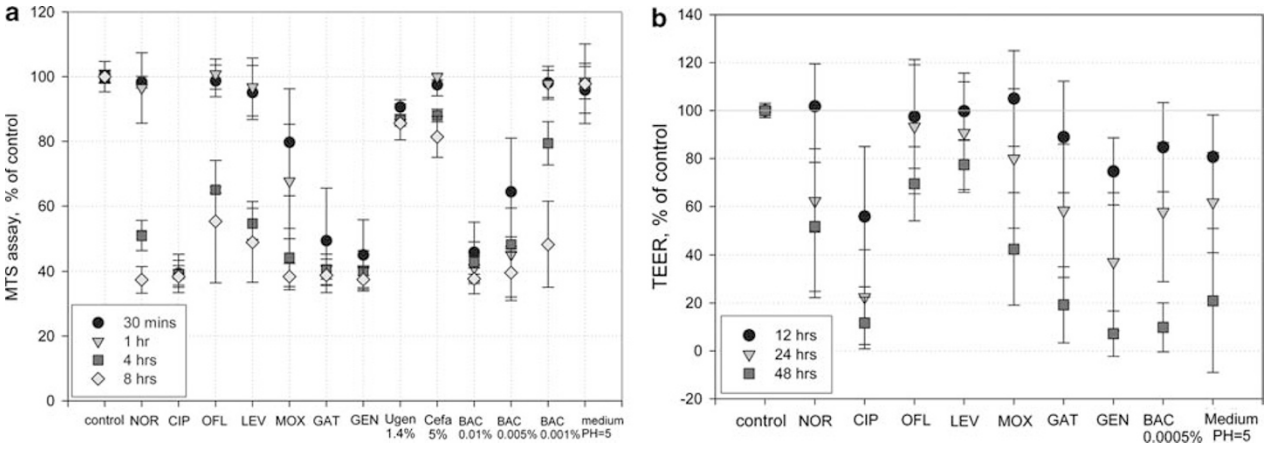

Figure 1 Comparison of cytotoxicity to HCECs from commercial preparations (a) MTS assay (% of control): HCECs incubated with commercial fluoroquinolone solutions, parental fortified gentamicin 1.4% and cefazolin 5%, benzalkonium chloride (0.01, 0.005, and 0.001%) and pH = 5 medium, compared with negative control in four time points. ($n = 12$, 4 replicates each of three independent experiments) (b) TEER (% of control), HCECs incubated in 1/10 concentration of commercial fluoroquinolone and gentamicin solutions, 0.0005% benzalkonium chloride and pH = 5 medium. After 12 h of incubation, ciprofloxacin induced a significant reduction of TEER compared with the other groups. After 48 h of incubation, TEER of gentamicin, 0.0005% BAC, ciprofloxacin, and gatifloxacin groups were significantly decreased. ($n = 9$, 3 replicates each of three independent experiments) ((NOR) norfloxacin, (CIP) ciprofloxacin, (OFL) ofloxacin, (LEV) levofloxacin, (MOX) moxifloxacin, (GAT) gatifloxacin, (GEN) gentamicin, (Ugen) parental fortified gentamicin, (Cefa) parental fortified cefazolin, (BAC) benzalkonium chloride).

Results

Cytotoxicity on HCECs: MTS assay (Table 1 and Figure 1a)

Commercial FQ and gentamicin ophthalmic solutions
Significant cell toxicity was found in the ciprofloxacin, gentamicin, gatifloxacin (Mann–Whitney rank-sum test, $P < 0.001$), and moxifloxacin groups (Mann–Whitney rank-sum test, $P = 0.01$) compared with the control group after a 30-min incubation. After 4 h, all the experiment groups showed a significant decrease in cell viability except for in levofloxacin and ofloxacin groups, and there was no difference between these two groups (*t*-test, $P = 0.399$). After 8 h, cells in all groups showed a significantly reduced viability compared with the control cells (Mann–Whitney rank-sum test, $P < 0.001$), with levofloxacin and ofloxacin showing relatively less cytotoxicity.

Commercial FQ and gentamicin ophthalmic solutions compared with preservative-free, parental fortified 1.4% gentamicin and 5% cefazolin

After 1 h, 5% cefazolin did not show significant cytotoxicity compared with ofloxacin and control (*t*-test, $P = 0.789$ and $P = 1.00$, respectively). The viability of cells treated with 1.4% gentamicin solution was significantly decreased compared with the control cells (Mann–Whitney rank-sum test, $P = 0.009$). However, the cytotoxicity induced by 1.4% gentamicin solution was significantly less than ciprofloxacin, gentamicin, and gatifloxacin ophthalmic solutions (*t*-test, $P < 0.001$). After 4 h, 1.4% gentamicin and 5% cefazolin showed less cytotoxicity towards HCECs than levofloxacin and ofloxacin (*t*-test, $P < 0.001$). Even after 8 h treatment, good cell viability was maintained in the 5% cefazolin and 1.4% gentamicin groups.

Commercial FQ and gentamicin ophthalmic solutions compared with BAC

Cells treated with 0.01, 0.005, and 0.001% BAC were incubated simultaneously with the other groups. After 30 min, 0.01 or 0.005% BAC showed significant cytotoxicity towards HCECs compared with the control group (Mann–Whitney rank-sum test, $P < 0.001$). However, 0.005% BAC induced less cytotoxicity than ciprofloxacin, gentamicin (*t*-test, $P < 0.001$, $P = 0.002$, respectively). The cytotoxicity induced by moxifloxacin was significantly less than that caused by 0.005% BAC at 1 h (*t*-test, $P = 0.005$). However, the difference was not significant after 4 h (*t*-test, $P = 0.482$).

Effects of groups of chemical-free medium at pH 5 and of distilled water, compared with negative control (chemical-free medium at pH 7.3)

There were no statistically significant differences in viability between cells incubated with chemical-free medium at pH 5 and at pH 7.3, as measured at five time points (30 min, 1–24 h). For HCECs cultured in media and in distilled water, significant cytotoxicity was found at 12 h (*t*-test, $P = 0.031$) between these two control groups.

Transepithelial electrical resistance

There were no statistically significant differences among testing groups in terms of TEER changes within the initial 8 h. (Table 2, Figure 1b) After 12 h, ciprofloxacin induced a significant reduction of TEER compared with the other groups. Ciprofloxacin and gentamicin induced significant decreases in TEER after 24 h of treatment, whereas levofloxacin and ofloxacin maintained TEER most of all. After 48 h, TEER measurements of the gentamicin, 0.0005% BAC, ciprofloxacin, and gatifloxacin groups were significantly decreased. Still, ofloxacin and levofloxacin retained the highest TEER (*t*-test, $P = 0.114$ between two groups). For cells treated with medium adjusted to pH 5, TEER was significantly decreased compared with the control group only after 48 h of incubation (*t*-test, $P = 0.012$).

Raw materials (standard powder) of FQs: MTS assay

After treating HCECs with pH adjusted, preservative-free 0.3% FQ solutions for 1 h, moxifloxacin and ciprofloxacin showed statistically significant toxicity compared with the control (Mann–Whitney rank-sum test, $P < 0.001$, $P = 0.005$, respectively) (Table 3, Figure 2). After 4 h, the viability of HCECs exposed to moxifloxacin, ciprofloxacin, gatifloxacin, and norfloxacin was significantly decreased (*t*-test, $P < 0.001$) with respect to the control. After 8 h, moxifloxacin showed the greatest cytotoxicity (*t*-test, $P < 0.001$) of all the groups

Table 2 TEER (% of control), HCECs incubated in the 1/10 concentration of commercial fluoroquinolone and gentamicin solutions, 0.0005% benzalkonium chloride and pH = 5 medium

	4 h (Mean ± SD)	*8 h (Mean ± SD)*	*12 h (Mean ± SD)*	*24 h (Mean ± SD)*	*48 h (Mean ± SD)*
Control	100.0 ± 2.31	100.0 ± 4.01	100.0 ± 3.12	100.0 ± 2.01	100.0 ± 1.58
0.03% Baccidal	103.73 ± 9.00	102.82 ± 24.09	101.77 ± 17.72	62.29 ± 40.08	51.60 ± 26.78
0.03% Ciloxan	90.87 ± 25.11	81.59 ± 33.04	55.84 ± 29.13	22.43 ± 19.77	11.66 ± 10.80
0.03% Oflovid	95.65 ± 18.19	107.93 ± 31.04	97.50 ± 21.60	93.29 ± 28.00	69.44 ± 15.45
0.05% Cravit	101.59 ± 22.74	101.77 ± 37.19	99.74 ± 12.20	90.74 ± 24.86	77.44 ± 10.40
0.05% Vigamox	98.42 ± 28.06	103.70 ± 30.84	105.00 ± 19.93	80.01 ± 29.08	42.41 ± 23.31
0.03% Zymar	105.81 ± 16.28	97.67 ± 32.16	88.96 ± 23.28	58.29 ± 27.68	19.21 ± 15.87
0.03% Garamycin	89.61 ± 8.01	90.64 ± 12.23	74.59 ± 13.99	37.07 ± 28.63	7.16 ± 9.46
0.0005% BAC	87.78 ± 17.35	95.32 ± 22.40	84.71 ± 18.62	57.79 ± 28.90	9.84 ± 10.19
pH = 5 medium	95.72 ± 16.55	84.89 ± 28.52	80.62 ± 17.53	61.73 ± 20.80	20.89 ± 29.91
P-value	0.05	0.257	< 0.001*	< 0.001*	< 0.001*

SD, standard deviation.
Kruskal–Wallis one-way analysis of variance on ranks, all pairwise multiple comparison (Dunn's method) in each time point. *P-value < 0.005 was considered as statistically significant. (Bonferroni-adjusted, $P < 0.05/10$).

Table 3 MTS assay (% of control): HCECs incubated with preservative-free 0.3% fluoroquinolone solutions compared with untreated cells (negative control)

	1 h (Mean ± SD) (P-*value*)	*4 h (Mean ± SD)* (P-*value*)	*8 h (Mean ± SD)* (P-*value*)	*24 h (Mean ± SD)* (P-*value*)
Negative control	100.00 ± 5.08	100.00 ± 3.56	100.00 ± 4.58	100.00 ± 2.40
0.3% Norfloxacin	100.88 ± 4.57 ($P = 0.730$)	91.55 ± 2.01 ($P < 0.001$)*	76.81 ± 8.34 ($P < 0.001$)*	36.24 ± 3.60 ($P < 0.001$)*
0.3% Ciprofloxacin	83.00 ± 15.67 ($P = 0.005$)$^{\#}$	75.50 ± 11.97 ($P < 0.001$)*	66.10 ± 23.30 ($P < 0.001$)*	39.69 ± 4.92 ($P < 0.001$)*
0.3% Ofloxacin	99.36 ± 5.12 ($P = 0.746$)	97.68 ± 7.18 ($P = 0.515$)	93.35 ± 9.10 ($P = 0.016$)$^{\#}$	68.68 ± 19.52 ($P < 0.001$)*
0.3% Levofloxacin	99.15 ± 6.02 ($P = 0.685$)	96.77 ± 7.14 ($P = 0.170$)	87.61 ± 8.79 ($P < 0.001$)*	47.67 ± 8.29 ($P < 0.001$)*
0.3% Moxifloxacin	82.58 ± 16.23 ($P < 0.001$)*	62.93 ± 11.23 ($P < 0.001$)*	48.74 ± 6.85 ($P < 0.001$)*	37.03 ± 1.95 ($P < 0.001$)*
0.3% Gatifloxacin	101.30 ± 6.43 ($P = 0.551$)	86.43 ± 8.02 ($P < 0.001$)*	70.42 ± 7.80 ($P < 0.001$)*	37.51 ± 3.24 ($P < 0.001$)*

SD, standard deviation.
Mann–Whitney rank-sum test, compared with negative control. *$P < 0.001$, $^{\#}P < 0.05$.
Negative control: HCECs in pH = 7.3 fresh medium without serum or antibiotics supplements.

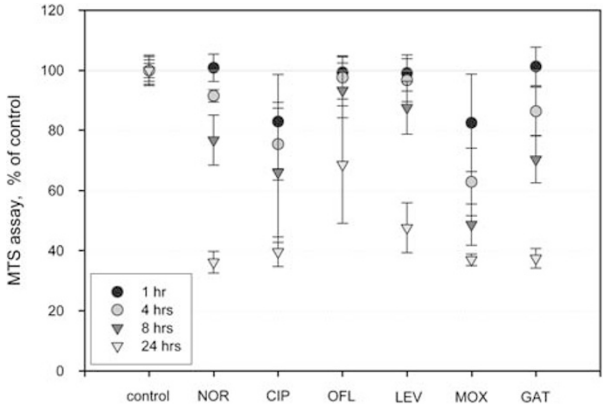

Figure 2 MTS assay (% of control): HCECs incubated with preservative-free 0.3% fluoroquinolone solutions compared with negative control. After 1 h of incubation, moxifloxacin and ciprofloxacin showed statistically significant toxicity. After 8 h of incubation, moxifloxacin showed the greatest cytotoxicity. After 24 h of incubation, HCECs treated with ofloxacin showed the best cell viability after 24 h incubation. ($n = 12$, 4 replicates each of three independent experiments) ((NOR), (CIP), (OFL), (LEV), (MOX), (GAT): same as Figure 1).

except for comparing with ciprofloxacin (Mann–Whitney rank-sum test, $P = 0.221$). HCEC cells treated with ofloxacin showed the best cell viability after 24 h of incubation. (Mann–Whitney rank-sum test, $P = 0.013$ with levofloxacin).

Cell morphology: inverted fluorescence microscopy

In a series of microscopy images, we observed that cells incubated with moxifloxacin detached from the culture plate within just 1 h and were completely suspended within 2 h. (Figure 3) A higher proportion of the cells remained viable in the ofloxacin and levofloxacin groups. At the end of the recording, the control group cells remained confluent and kept in cuboid form, which was similar to primary cultured corneal epithelial cells.

Discussion

The epithelium is the main barrier of the cornea. The level of topical drug penetration varies up to 20-fold, depending on the integrity of the corneal epithelium.[29] Owing to the corneal epithelial barrier, the eye is relatively impermeable to microorganisms and other environmental elements. The healing of epithelial defects is also an important indicator that bacterial keratitis has been effectively treated, as well as being a crucial factor that affects visual outcome after refractive surgery. To eradicate the bacteria during infectious keratitis, topical medications should be given at a very high frequency. Therefore, it is important to choose an appropriate topical medication, which has the least cytotoxicity.

In the initial stages of our study, we used the MTS assay to evaluate cell viability after treating cells with commercial ophthalmic solutions. Cell viability decreased dramatically in the ciprofloxacin, gentamicin, and gatifloxacin groups after only 30 min of incubation. The most significant difference in the formulation of these three commercial eye drops from the other FQs is the presence of BAC. BAC is the most frequently added preservatives in ophthalmic solutions. Its cytotoxic effect to corneal and conjunctival cells had been established in earlier studies.[30,31] To further support our studies, we used HCECs cultured in different concentrations of BAC to compare HCECs in FQ and in gentamicin solutions. In the first 30 min, the commercial ciprofloxacin and gentamicin solutions showed greater cytotoxicity than 0.005% BAC alone. However, cell viability in these three groups plus gatifloxacin group showed no difference after 1 h of treatment. We could presume that other factors such as pH and the chemical structures of the drugs aggravated the cytotoxicity besides the 0.005% BAC present in commercial ciprofloxacin and gentamicin solutions.

The stratified corneal epithelium consists of 5–7 layers of cells, with tight junctions (TJs) at the superficial layer.[32]

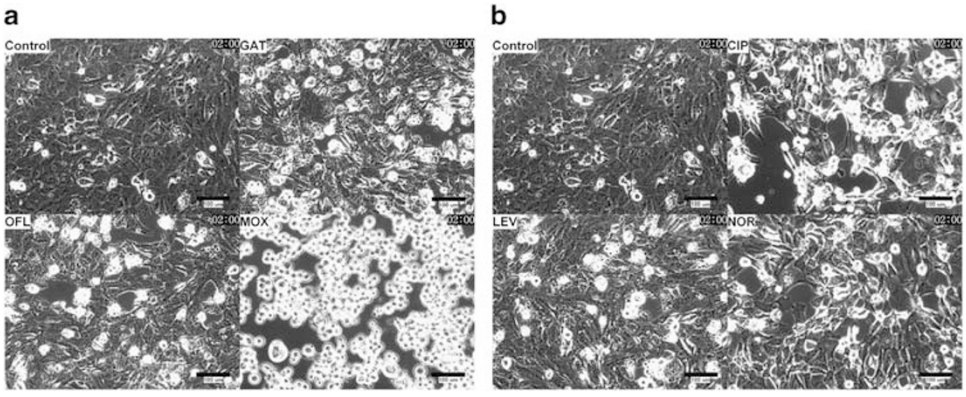

Figure 3 HCECs treated with preservative-free 0.3% FQ solutions. The images were taken from inverted fluorescence microscopy with multiple stage position, in time-lapse mode. After 2 h, HCECs treated with moxifloxacin had all detached from the culture well (a). Cells treated with norfloxacin and ciprofloxacin had become elongated in shape (b). ((NOR), (CIP), (OFL), (LEV), (MOX), (GAT): same as Figure 1).

Loosening of TJs may be an early change in the epithelial barrier followed by destructive events caused by drug toxicity. Immortalized HCECs grown on filters, using various filter materials and coating procedures, could be used as an *in vitro* model that mimics the intact cornea.[33,34] A decrease of TEER indicates an increase in paracellular permeability, which represents the disruption of TJs. HCECs died and detached from the wells rapidly after treatment with commercial FQ solutions in the MTS assay. Therefore, we diluted the solutions to 1/10 the concentration to better observe changes in TEER. Similar to the results of the MTS assay, TEER decreased most evidently in the ciprofloxacin and gentamicin groups during the initial stages. After 48 h, those incubations containing BAC gave the lowest TEER values. To elucidate the role of BAC and to compare the cytotoxicity of the distinct chemical characteristics of the different FQs, we used the standard antibiotic powders and the MTS assay for testing. The viability of cells treated with moxifloxacin decreased most rapidly among all the FQs tested in the initial 8 h. Ofloxacin showed less cytotoxicity. Our results suggest that there are differences in the intrinsic cytotoxicity of the various FQs, quite separate from the influence of additives and pH.

The most common fortified antibiotics used to treat bacterial keratitis are 1.4% gentamicin and 5% cefazolin. Some earlier studies compared the cytotoxicity between FQs, ofloxacin and the aminoglycoside, netilmicin.[35–37] They concluded that netilmicin was significantly less toxic than ofloxacin to human keratocytes,[37] to intact and mechanically damaged rabbit eyes,[36] and to rabbit corneal epithelial cells.[35] Mallari *et al*[38] also found an increased risk of corneal perforation after FQ treatment for bacterial keratitis, compared with treatment with fortified antibiotics (cephazolin 5% and tobramycin 1.36%).In our study, preservative-free fortified gentamicin and cefazolin were less cytotoxic to corneal epithelium cells than all the other FQ solutions tested.

Regarding pH, norfloxacin and ciprofloxacin need to be dissolved in a weakly acidic environment (around pH 6.0, 5.0, respectively). From our results, the HCECs cultured in a low pH environment (pH = 5) did not show significant cytotoxicity over the control cells (pH = 7.3) even after 24 h of incubation. A low pH environment might pose an adverse influence on cell growth and may account in part for the cytotoxic effects of norfloxacin and ciprofloxacin. However, the effect was not significant compared with other factors such as preservatives and chemical drug structures *per se*. Although our experiments did not show that pH had a significant influence on the viability of HCECs, the more neutral environment in tear film might cause the precipitation of norfloxacin and ciprofloxacin, which may delay epithelial healing of ulcerative keratitis and result in corneal perforation.[39–41]

Numerous studies have compared the cytotoxicity of different FQs, but the results remain controversial. Burka *et al*[21] compared the epithelial wound healing after the treatment of photorefractive keratectomy with moxifloxacin (Vigamox) or with gatifloxacin (Zymar), and found that the eye treated with moxifloxacin healed faster. Kovoor *et al*,[22] using confocal microscopy to evaluate corneal epithelium and stromal thickness after treating rabbits with different FQs, found that moxifloxacin induced less damage than other FQs. However, the ofloxacin and levofloxacin they tested contained 0.005% BAC, which was different from the commercial solutions we tested. Another study, using confocal microscopy and ZO-1 staining on rabbit eyes, also showed that moxifloxacin resulted in better corneal epithelial integrity and TJ organization than gatifloxacin did, after a short-term, intensive exposure.[23] Kim *et al*[19]

found levofloxacin (0.5%, Cravit; Santen Pharmaceutical Co.) was less toxic than moxifloxacin (0.5%, Vigamox; Alcon Laboratories Inc.) to corneal epithelial cells, using the MTT assay and by measuring the rate of wound healing *in vitro*. The levofloxacin they tested was a preservative-free formulation, the same as the one we used. Matsumoto *et al*[42] used the standard powders of FQs to compare them and found that moxifloxacin was a stronger inhibitor of corneal epithelial cell migration than gatifloxacin at the same concentration, at high dose. They ranked FQs in order of decreasing inhibition of cell proliferation for HCECs as ciprofloxacin = moxifloxacin > gatifloxacin > levofloxacin > ofloxacin = untreated, which is similar to our results. In our study, we used both commercial ophthalmic solutions and standard FQ powders for comparison. We also compared the fortified, parental antibiotics with commercial FQ drops, as well as the preservative BAC and the effects of using a lower pH medium, in the hopes of better clarifying the source of cytotoxicity caused by FQs. The limitation of our study was the *in vitro* setting and the use of immortalized HCEC line instead of primary cultures of corneal epithelial cells. However, owing to the scarcity of available tissue and restricted lifespan of primary cultures, immortalized cell lines is a practical tool for toxicity screening of ocular drugs with good sensitivity and reliability.[43] The results could serve as a reference for clinical application.

In conclusion, our study showed that the main source of cytotoxicity from commercial FQ ophthalmic solutions came from the preservatives. Gentamicin and cefazolin, without any preservatives, were less toxic to the corneal epithelium, even when applied at a fortified concentration. The intrinsic toxicity of FQs towards HCECs still varied, regardless of any preservatives present. In our study, fourth-generation FQs were not less toxic to HCECs than the older generation ones.

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