

EVALUATION OF POLYQUATERNIUM-1 EFFECTS ON HUMAN CORNEAL EPITHELIAL CELLS

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Manuscript received: January 2017

Abstract

The aim of this study was to evaluate the antibacterial activity of polyquaternium-1 and its toxicity on human corneal epithelial cells. The antibacterial activity of polyquaternium-1 (PQ-1) samples was measured by nutrient broth dilution method, and the influence of external factors on the stability of PQ-1 was investigated. The cytotoxicity of polyquaternium-1 was evaluated using an *in vitro* cell model of human corneal epithelium HCEpiC. The PQ-1 samples had a good antibacterial activity, which was not affected by UV irradiation and pH. The antibacterial activity decreased with the increase of temperature and NaCl. In addition, the PQ-1 high concentration had certain toxic effects on HCEpiC, but the cytotoxicity of PQ-1 was low compared with other tested antibacterial agents. Polyquaternium-1 is a good choice as an ophthalmic bacteriostatic agent, being non-irritant and having strong antibacterial activity, low toxicity, and low cytotoxicity on human corneal epithelial cells.

Rezumat

Obiectivul studiului a fost de a evalua activitatea antibacteriană și toxicitatea compusului polyquaternium-1 (PQ-1) asupra celulelor epiteliale corneene umane. A fost evaluată activitatea antibacteriană a probelor de PQ1 prin metoda diluțiilor și influența diferiților factori externi asupra stabilității acestuia. Citotoxicitatea PQ-1 a fost analizată utilizând un model *in vitro* de celule epiteliale corneene umane HCEpiC. Probele preparate au prezentat o bună activitate antibacteriană, activitate care nu a fost afectată de radiațiile UV și pH. Activitatea antibacteriană a scăzut odată cu creșterea temperaturii și a concentrației de NaCl. La concentrații mari, PQ-1 a prezentat anumite efecte toxice asupra HCEpiC, dar acestea au fost mult mai mici comparativ cu agenții antibacterieni luați în studiu. PQ-1 este o alegere bună ca agent bacteriostatic oftalmologic, prezentând efecte non-iritative, o activitate antibacteriană puternică și o toxicitate redusă, cu efecte citotoxice scăzute asupra celulelor epiteliale corneene umane.

Keywords: polyquaternium-1, corneal epithelial cells, cytotoxicity, antibacterial activity

Introduction

Ophthalmic preparations refer to sterile biological products directly used for eye treatment infections [23] that occur in different situations, such as: patients with severe acne refractory to the classical treatment [17], immunosuppressed patients with mixed bacterial and fungal skin infections, especially with fungus from *Fusarium* genus [32], patients with complicated surgical site infections [5, 30], patients with pneumonia complicated with parapneumonic pleurisy accompanied by purulent sputum due to superinfection with opportunistic pathogens that can accidentally get to conjunctival mucosa [6], patients from intensive care unit carrying multidrug resistant bacteria that can cause severe ocular bacterial conjunctivitis difficult to treat [10], or patients with different ocular infectious diseases [12]. In recent years, the report of the toxicity of ophthalmic antibacterial agents increased and people gradually paid attention to their safety and rationality. The

common ophthalmic antimicrobial agents are divided into quaternary ammonium salts, alcohols, organic mercury derivatives, hydroxyphenyl esters and acids [27, 29, 31]. Every kind of bacteriostatic agent has different antibacterial mechanisms of action.

Sometimes, in order to improve the antibacterial activity and bactericidal ability, mixtures of antibacterial agents are used [2]. The long-term use of ophthalmic preparations and other drugs could cause different degrees of injury to the human corneal epithelium, corneal endothelium, iris and ciliary body and other ocular tissues [1, 13, 14]. There are studies that showed that about 12% of conjunctivitis were iatrogenic [16, 22]. The most used bacteriostatic agents in ophthalmic preparations are quaternary ammonium salts such as benzalkonium chloride (BAC). There are studies that show that benzalkonium chloride can lead to eye inflammation, allergies, as well as the dry eye and other adverse reactions [4, 15, 25]. Debbash *et al.* [11] tested

benzalkonium chloride *in vitro* on cell cultures and showed that it has a clear cytotoxicity, in usual concentrations and below. It can cause conjunctival cells damage, and in high concentration conjunctival cell necrosis. Polyquaternium (PQ-1) [20] is a strong cationic high-molecular polymer. This new type of bacteriostatic agent seems to have fewer harmful effects on human corneal epithelial cells. In this study we aimed to assess the toxic effect of polyquaternium-1 used as a bacteriostatic agent on human corneal epithelial cells.

Materials and Methods

Reagents

The used reagents were: Polyquaternium-1 reference substance (Suzhou Yake Technology Co., Ltd, batch: BK10-PW-P01); *Staphylococcus aureus* ATCC 21923 (Shanghai Bio Excellence Co. Ltd.); *Pseudomonas aeruginosa* ATCC 31554 (Shanghai Bio Excellence Co, Ltd); *Escherichia coli* ATCC 25922 (Shanghai Bio Excellence Co, Ltd); *Candida albicans* ATCC 28366 (Shanghai Bio Excellence Co, Ltd); *Aspergillus niger* ATCC 16620 (Shanghai Bio Excellence Co, Ltd); nutrient agar medium (Shanghai Bio Excellence Co., Ltd.); potato dextrose agar (PDA medium (Shanghai Bio Excellence Co., Ltd.); nutrient broth medium (Nanjing Bianzhen Bio Tec CO., Ltd.); benzalkonium chloride (Shandong Hua Mei Water Treatment Environmental Protection CO., Ltd.); ethylparaben (Wuhan Qian Run Bioengineering CO., Ltd); modified RPMI-1640 medium (Nanjing Bianzhen Bio Tec CO., Ltd.); foetal calf serum (Shanghai Serum Biotechnology Co., Ltd); trypsin (Shanghai Serum Biotechnology Co., Ltd); human corneal epithelial cell line 6510HCEpiC (Beijing Yu Heng Feng Technology Co., Ltd.); lactate dehydrogenase (LDH) kit (Nanjing Sen Bei Jia Biotechnology Co., Ltd.); live - dead cell staining kit and other reagents (Biovision Company in US).

Equipment

The main equipment used: lyophilizer (Beijing Yong Guangming Medical Equipment Factory); pipette (Hangzhou Loh and Biological Co., Ltd.); rotary evaporator (Beijing Yong Guangming Medical Equipment Factory); SHB-III circulating water-type multi-purpose vacuum pump (Shanghai Ying Di Instrumentation Equipment Co., Ltd.); nuclear magnetic resonance system (NMR) (Shanghai Aidu Energy Science and Technology Co., Ltd.); Nicolet IR200 Fourier transform infrared spectrometer (Shanghai Fairborn Industry Development Co., Ltd.); dialysis bag (Hefei Bo Mei Biotechnology Co. Ltd).

Methods

Determination of antibacterial and antifungal activity and stability of polyquaternium-1

Fresh cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*

and *Aspergillus niger* were used. Suspensions of 10^8 CFU/mL were prepared using sterile saline solution. 0.1 mL from the above suspensions were added to 10 mL sterile saline, and the final concentration was about 10^6 CFU/mL. Polyquaternium-1 solutions with concentrations of 1300 and 1000 µg/mL were prepared. The minimum inhibitory concentration (MIC) of polyquaternium-1 was determined by nutrient broth dilution method. Briefly 8 sterile test tubes were used. 0.4 mL polyquaternium-1 sample solution was added into a tube and then, serial dilutions were made accordingly. Afterwards in each tube was added 1 mL 10^6 CFU/mL of the tested germs suspension so that the final concentration in each tube was approximately 5×10^5 CFU/mL. Samples were incubated at 37°C for 20 h. The experiment was repeated five times. The minimal inhibitory concentration (MIC) of the antimicrobial agent was considered the concentration of the antimicrobial agent from the highest dilution tube that presented sterile growth (clarification) compared to control tube (corrosion) [28, 34, 35]. The MBC (minimum bacterial concentration) was also assayed.

The effects of different conditions such as UV irradiation, temperature, NaCl concentration and pH on the antibacterial activity of polyquaternium-1 were also assayed and each experiment was repeated five times.

Determination of cytotoxicity of PQ-1 on human corneal epithelial cells cultured *in vitro*

The 6510HCEpi was incubated in a cell incubator (5% CO₂, 37°C, 95% humidity) to reach 80% confluence of the cells. The original culture medium was discarded and the test solutions were added and incubated for 10 min, 20 min, 60 min, and 120 min, respectively, then the assessment of cytotoxicity was carried out. The PQ-1 stock solution was diluted using RPMI-1640 medium to 0.01%, 0.005%, 0.001%, and 0.0005%, respectively. At the same time, 0.01% benzalkonium chloride and 0.03% ethylparaben solution were prepared, and stored after sterile filtration. The normal control group consisted in cells cultured in the medium without any drug.

The morphological changes of HCEpiC cells in the control and treatment groups were observed through an inverted microscope. The activity of HCEpiC was detected using a living-dead cell staining kit, and the survival rate of cells was calculated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. At each time point the suspensions were centrifuged (3000 rpm, 5 min) and the supernatant was used for the assay of lactate dehydrogenase (LDH) activity.

Statistical methods

SPSS17.0 statistical software was used for analysis. All data formats were shown as $\bar{x} \pm s$ and Student t test was conducted to compare data. There was statistical

significance for variation analysis of data among groups when $p < 0.05$.

Results and Discussion

Antibacterial activity and stability of polyquaternium-1

The Table I shows the MIC and MBC values of polyquaternium-1 for the five common pathogens.

As can be seen from Table I, the MICs of PQ-1 for *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger* were 16 $\mu\text{g/mL}$, 4 $\mu\text{g/mL}$, 16 $\mu\text{g/mL}$, 8 $\mu\text{g/mL}$ and 32 $\mu\text{g/mL}$ respectively, and the MBCs of the five pathogens were 16 $\mu\text{g/mL}$, 8 $\mu\text{g/mL}$, 32 $\mu\text{g/mL}$, 8 $\mu\text{g/mL}$ and 64 $\mu\text{g/mL}$, respectively.

Table I

MIC and MBC values of polyquaternium-1 for the five pathogens

PQ-1 concentration ($\mu\text{g/mL}$)	<i>Staphylococcus aureus</i> ATCC 21923		<i>Escherichia coli</i> ATCC 25922		<i>Pseudomonas aeruginosa</i> ATCC 31554		<i>Candida albicans</i> ATCC 28366		<i>Aspergillus niger</i> ATCC 16620	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
2	T	—	T	—	T	—	T	—	T	—
4	C	2.88	T	—	T	—	T	—	T	—
8	C	4.50	T	—	T	—	C	4.11	T	—
16	C	5.09	C	3.28	C	2.11	C	4.69	T	—
32	C	$+\infty$	C	5.71	C	3.79	C	5.68	C	—
64	C	$+\infty$	C	$+\infty$	C	4.40	C	$+\infty$	C	3.31
128	C	$+\infty$	C	$+\infty$	C	$+\infty$	C	$+\infty$	C	3.52
Contrast	T	—	T	—	T	—	T	—	T	—

Notes: "T" = turbidity of the culture medium; "C" = the clarification of the culture medium; data is the logarithmic decrease of the relative initial number of bacteria.

The results of several other treatment conditions (UV irradiation, temperature, NaCl concentration, pH) on the stability of the antibacterial activity of polyquaternium-1 are shown in Figures 1 to 4.

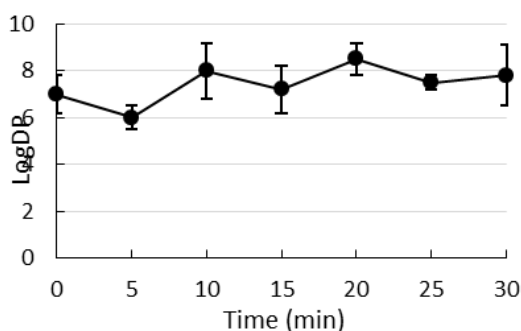


Figure 1.

Effect of ultraviolet irradiation time on the antibacterial activity of PQ-1

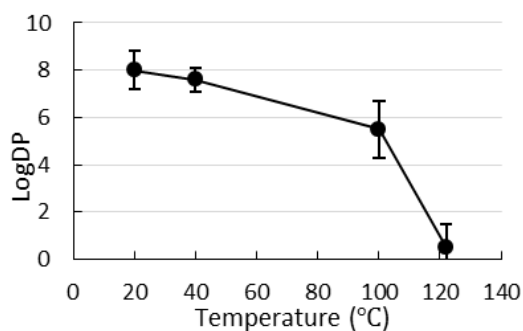


Figure 2.

Effect of temperature on the antibacterial activity of PQ-1

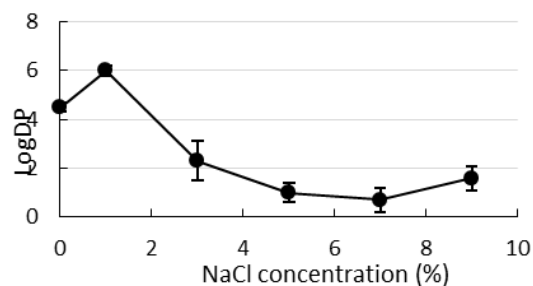


Figure 3.

Effect of salt concentration on the antibacterial activity of PQ-1

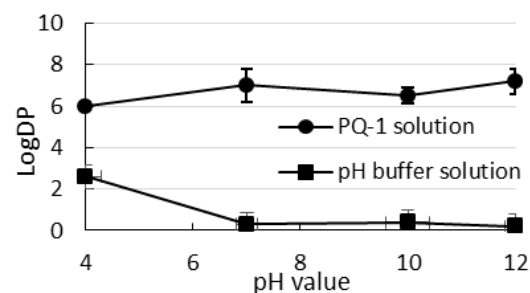


Figure 4.

Effect of pH on the antibacterial activity of PQ-1

It can be seen from Figure 1 and Figure 4 that the antibacterial activity of polyquaternium-1 remained almost constant with the change of irradiation time and pH, indicating that PQ-1 had good stability. It can be seen from Figure 2 that the antibacterial activity of PQ-1 decreased gradually with the increase of temperature. When the temperature reached 120°C, its antibacterial activity was almost lost. Therefore, PQ-1 showed high instability to heat.

It can be seen from Figure 3 that the antibacterial activity of PQ-1 was the best when the concentration of NaCl was 1%, but the antibacterial activity of PQ-1 also decreased with the increase of NaCl concentration.

Cells morphological changes

In the control group, HCEpiC cells were morphologically intact and arranged in a neat and

clear outline. Using 0.01% PQ-1, 0.005% PQ-1 and 0.01% benzalkonium chloride, the cells were obviously deformed, they were shrunk and became circular, and the refraction was weakened.

Moreover, with the prolongation of the acting time, the changes in cell morphology became more obvious (Figure 5).

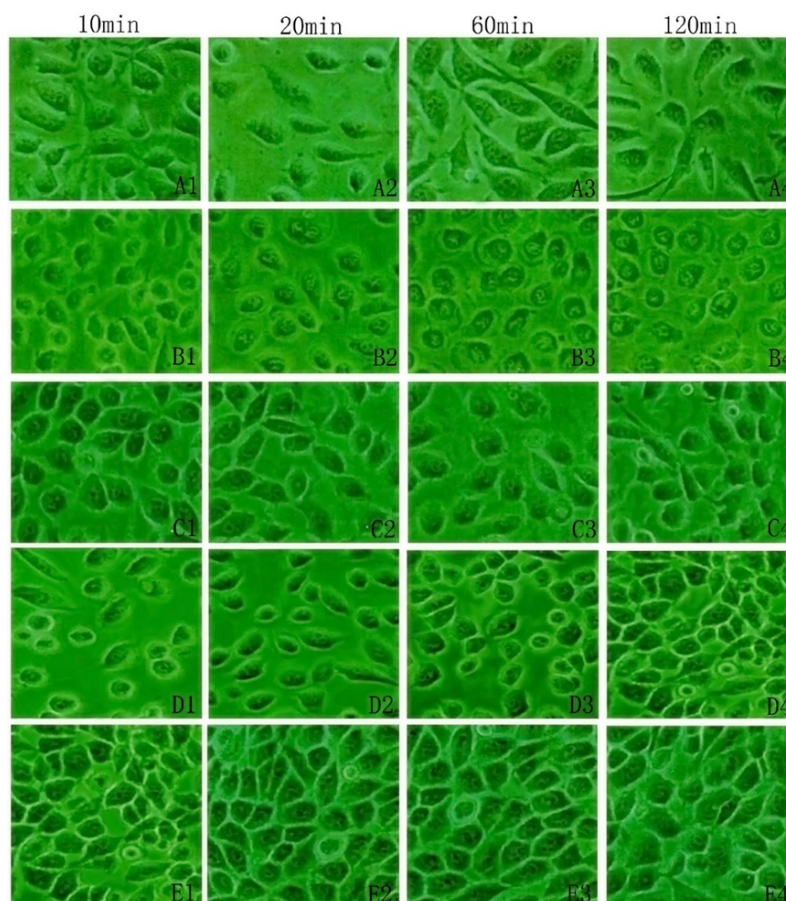


Figure 5.

HCEpiC control cells morphology (A1 ~ A4), 0.01% benzalkonium chloride cell morphology (B1 ~ B4), 0.01% PQ-1 cells morphology (C1 ~ C4), 0.03% ethylparaben cell morphology (D1 ~ D4), 0.005% PQ-1 cells morphology (E1 ~ E4)

Cell survival rate

If the cell survival rate of the control group was 100%, it can be seen from the data in Table II that the cell survival rate of 0.01% PQ-1, 0.005% PQ-1

and 0.01% benzalkonium chloride was lower ($p < 0.05$). The decreased speed of cell survival rate increased with time, and the cell survival rate of 0.01% benzalkonium chloride group was the lowest.

Table II

The absorbance values of MTT in each group ($\bar{x} \pm s$, $n = 6$)

Groups	10 min	20 min	60 min	120 min
Control group	0.828 ± 0.051	0.875 ± 0.036	0.958 ± 0.078	0.998 ± 0.102
0.01% PQ-1	$0.441 \pm 0.018^*$	$0.412 \pm 0.041^*$	$0.263 \pm 0.020^*$	$0.180 \pm 0.031^*$
0.005% PQ-1	$0.641 \pm 0.048^*$	$0.560 \pm 0.020^*$	$0.447 \pm 0.063^*$	$0.291 \pm 0.087^*$
0.001% PQ-1	0.846 ± 0.076	0.873 ± 0.120	0.915 ± 0.029	1.023 ± 0.165
0.0005% PQ-1	0.844 ± 0.068	0.896 ± 0.103	0.957 ± 0.135	1.101 ± 0.109
0.01% benzalkonium chloride	$0.312 \pm 0.011^*$	$0.283 \pm 0.032^*$	$0.168 \pm 0.010^*$	0.142 ± 0.009
0.03% ethylparaben	0.802 ± 0.032	$0.742 \pm 0.101^*$	$0.731 \pm 0.083^*$	$0.758 \pm 0.069^*$

Notes: * $p < 0.05$ versus the control group

LDH activity

LDH activity reflects changes in the cell membrane permeability, and it is usually used to evaluate the cytotoxic effect of drugs. LDH activity results are shown in Figure 6. The LDH activity increased continuously with the antibacterial effect, in time.

The increase of LDH activity was more significant with the increase of the PQ-1 concentration. Three types of bacteriostatic agents were compared and the increase of LDH activity was the lowest in 0.03% ethylparaben - treated group.

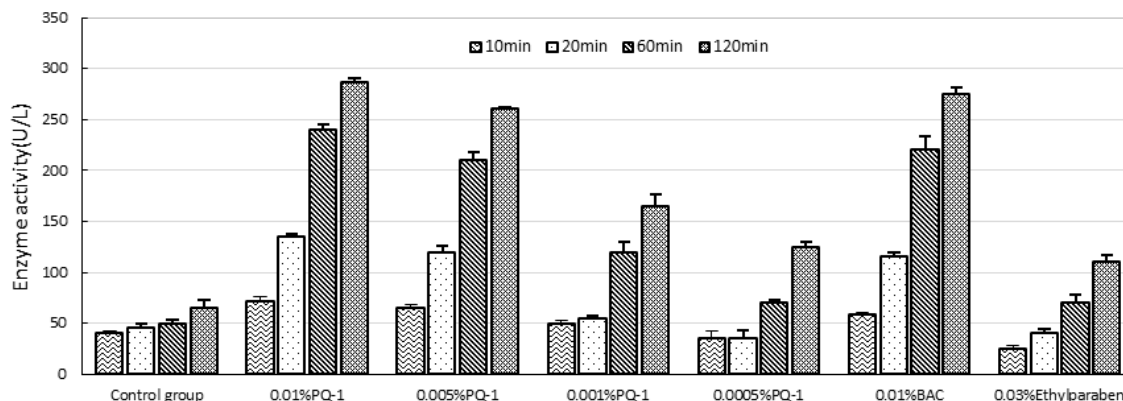


Figure 6.

The test results of LDH activity at different time points for each group

Cell viability

The cell viability of HCEpiC in the control group was the highest, with a cell survival rate close to 100%. A small number of HCEpiC cells survived after 10 min exposure to 0.01% PQ-1 and 0.005% PQ-1, but died after 20 min. 0.01% benzalkonium

chloride showed lower cell viability at all time points, and almost no cell survived. The cell viability of the other groups was relatively good, but the death rate increased after exposure to 0.03% ethylparaben for 120 min. Partial cell activity test results are shown in Figure 7.

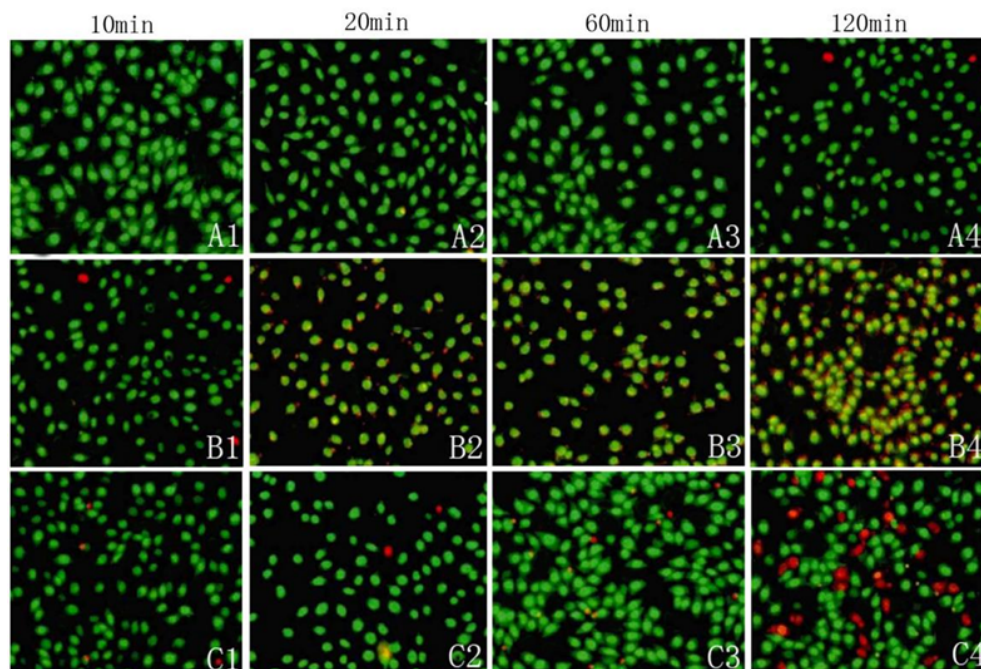


Figure 7.

Cell viability assay (control group: A1 to A4, 0.01% PQ-1: B1 to B4, 0.03% ethylparaben: (C1 to C4))

Considering the global perspective of our study results, several aspects should be discussed. The stability results of PQ-1 showed that UV irradiation

and pH had no significant effect on the antibacterial activity of PQ-1. However, with the increase of the temperature, the antibacterial activity showed a

decreasing trend. Because for some polymer-based bacteriostatic agents, the thermal stability is poor, and easy to degrade under high temperature conditions, the antibacterial activity of antimicrobial agents decreased [24]. Therefore, in the course of PQ-1 use, we should avoid heating. When the concentration of NaCl was 1%, the antibacterial activity of polyquaternium-1 was the best, which may be related to its antibacterial mechanism [21]. In the physiological salt concentration system, the cell morphology is the best, which favours PQ-1 to penetrate the cell wall, and exert the antibacterial effect [26, 33]. The results indicate that PQ-1 acts as bacteriostatic at low concentrations, so it has a potential as antibacterial agent for ophthalmic use. The common mass concentration of PQ-1 is 0.001% [9]. The animal studies show that [3, 18, 19] high concentrations of polyquaternium-1 (0.1% and 0.5%) diminish the goblet cell density significantly, but do not cause damage to the conjunctival tissue, and its effect on the amount of tear secretion is also not significant. Other studies [7, 8] have shown that two kinds of contact lens care solution that have PQ-1 as antibacterial agent possess a certain corneal cytotoxicity. In our study, the cytotoxicity of different concentrations of PQ-1 was evaluated by establishing an *in vitro* cell model of HCEpiC compared to the cytotoxicity of other ophthalmic preparations containing bacteriostatic agents: benzalkonium chloride and ethylparaben. The results showed that PQ-1 with high concentration has a potent cytotoxic effect on HCEpiC which is time-dependent. PQ-1 at the usual concentration and has no toxic effect on HCEpiC, with no significant difference compared to the control group. Regarding the other bacteriostatic agents, benzalkonium chloride showed the highest cytotoxicity. Although the toxicity of ethylparaben is low, its solubility is also low, the antimicrobial spectrum is narrow and the antibacterial effect on *Pseudomonas aeruginosa* was weak.

Conclusions

PQ-1 proved to be good choice as an ophthalmic antimicrobial agent. Compared to other ophthalmic antimicrobial agents, it is active at low concentrations, possesses potent antibacterial effect, and small side effects, and its effect on the corneal epithelial cells is not significant, indicating that the use of PQ-1 in ophthalmic preparations is safe and feasible.

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