Inhibitory Effect of Diclofenac Sodium on the Proliferation of Rabbit Corneal Epithelial Cells in vitro

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Purpose: To investigate the inhibitory effect of diclofenac sodium on rabbit corneal epithelial cells (RCECs) in vitro and explore its pharmacological mechanism.

Methods: The fresh rabbit cornea was cultured to get the primary RCECs, and RCECs of passage 2 were used for the research. The cells were divided into experimental groups, the cells in which were incubated with different concentrations (18.18, 27.27, 36.36, 45.45, 54.55 μg/ml) of diclofenac sodium, and control group. The effect of diclofenac sodium on the proliferation of cells was measured by methyl thiazolyl thiazolium (MTT) assay 24, 48 and 72 h after incubation. While the RCECs were divided into experimental groups, the cells in which were incubated with 9 and 12.5 μg/ml diclofenac sodium, and control group. The cell cycle and apoptotic rate were observed by flow cytometer.

Results: MTT assay showed that diclofenac sodium had obvious inhibitory effect on RCECs, and the inhibition rate was increasing along with the increase of the concentration of diclofenac sodium and the incubation time ($P<0.05$). Flow cytometer showed that after incubation with diclofenac sodium, the cells in G_0/G_1 phase were obviously increased, the apoptosis cusp and apoptotic rate were increased.

Conclusion: Diclofenac sodium has obvious inhibitory effect on RCECs, which was dosage-dependent, and it may function by inducing cell apoptosis and ceasing cells cycles.

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Key words: Diclofenac sodium; Corneal epithelial cells, Rabbit; Inhibition

Myopia is the most common ametropia in the world. The occurrence rate is over 25% in United State and 70% in Asia. As a method of treating myopia, laser in situ keratomileusis (LASIK) has been accepted by many people because of its stability, predictability and safety. Corneal epithelial ingrowth is a common complication after LASIK, and the occurrence rate is about 20%[1]. It may result in astigmatism, low vision, dizzy and the corneal flap melting[2]. So how to decrease the occurrence rate of corneal epithelial ingrowth has become a focus concerned by many ophthalmologists.

It is reported that diclofenac sodium can inhibit fibroblast cells[3], but the effect of di clofenac sodium on epithelial cells is unclear. By culturing corneal epithelial cells in vitro, the study aims to observe the effect of diclofenac sodium on corneal epithelial cells and to explore the pharmacological mechanism.

Materials and methods

Materials and animals

Diclofenac sodium (Wuhan Wujing Pharmaceutical factory); Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/F12) (Hyclone, U.S.A); Fetal calf serum (Hangzhou Sijiqing Biological Engineering Company); TACS Calibur Flow Cytometer (Becton-Dickinson company, USA); New Zealand white rabbits weighing 1.8~2.5 kg (Experimental Animal Center of Chongqing Medical University).

Culture of rabbit corneal epithelial cells (RCECs)

Healthy New Zealand white rabbits were killed and the corneas were removed. The endothelial surface was wiped off, and the epithelial surface was scored gently by 5# needle. The corneas were cut into 4~6 pieces and then put into a 25 cm^2 tissue culture flask which was cultured in a 37℃ incubator with 5% CO_2. The culture media was replaced 2 or 3 times a week. Six hours later the cells began to emigrate from the tissue. The epithelial cells grew to a confluent monolayer about 8 days later, and it was the right time to passage.

Detection of inhibitory effect of diclofenac sodium on RCECs

The RCECs of passage 2 were divided into six groups: different concentrations of diclofenac sodium groups and control group. RCECs in diclofenac sodium groups were incubated with DMEM/F12 containing 18.18, 27.27, 36.36, 45.45, 54.55 μg/ml diclofenac sodium, respectively. The cell proliferation was measured with methyl thiazolyl tetrazolium (MTT) assay 24, 48 and 72 h after incubation and absorbance A value was read. The inhibition rate was calculated according to the formula: inhibition rate =1 -average absorbance A value of experimental group/average absorbance A value of control group ×100%. IC_{50} was calculated based on regression equation.

Detection of cell cycle of RCECs

The RCECs were divided into 3 groups: two experimental groups and control group. The cells in experimental groups were incubated with DMEM/F12 containing 9 and 12.5 μg/ml diclofenac sodium, respectively, while the cells in control group were cultured with DMEM/F12 only. The cell cycle and apoptotic rate were analyzed by flow cytometer 48 h later.

Statistical analysis

All data were expressed as Mean±SD and analyzed with SPSS10.0 software. The differences among groups were tested by ANOVA or χ^2 test, and the pairwise comparison were tested by Least-significant-difference (LSD), and it was considered significant if $P<0.05$.

Results

1 The inhibitory effect of diclofenac sodium on RCECs

MTT assay showed that diclofenac sodium had obvious inhibitory effect on RCECs. At 24 and 48 h, A value descended obviously with the increasing concentrations of diclofenac sodium. The inhibition rate was increasing with the expansion of incubation time in 18.18, 27.27, 36.36, 45.45 μg/ml groups (Tab 1).

2 Chang of the cell cycle after treatment of diclofenac sodium

There were few apoptosis cells in the control group and obvious apoptosis cells in the experimental groups. After the incubation of diclofenac sodium, the cells in G_0/G_1 phase increased, while in S phase decreased, and there was significant difference among the three

Tab.1 The inhibited effect of diclofenac sodium on corneal epithelial cells ($\bar{x}\pm s$)

groups	24 h	48 h	72 h
	A value[inhibitory rate(%)]	A value[inhibitory rate (%)]	A value[inhibitory rate (%)]
Control group	0.057±0.006(-)	0.084±0.022(-)	0.100±0.025-
18.18 μg/ml	0.078±0.016(-)*	0.044±0.007 (47.6)*	0.046±0.003(54.0)*
27.27 μg/ml	0.033±0.006(42.1)*	0.025±0.001(70.2) *	0.005±0.004(95.0)*
36.36 μg/ml	0.022±0.009(61.4)*△	0.016±0.007 (81.0)*△	0.005±0.002(95.0)*
45.45 μg/ml	0.018±0.010(68.4)*△▲	0.007±0.002(91.7)*△▲	0.005±0.004(95.0)*
54.55 μg/ml	0.006±0.002(89.5)*△▲▽	0.006±0.003(92.9)*△▲	0.008±0.001(92.0)*△▲▽

Note: * Compared with the control or 18.18 μg/ml group, $P<0.05$; △Compared with 27.27 μg/ml group, $P<0.05$; ▲Compared with 36.36 μg/ml group, $P<0.05$; ▽Compared with 45.45 μg/ml group, $P<0.05$; Except control and 54.55 μg/ml group, the differences between every two time points were significant ($P<0.05$) in other groups; IC_{50} of 48 h is 18.12 μg/ml

groups ($P<0.01$). The apoptotic rates were significantly different among three groups ($P<0.01$), and it showed that the apoptosis cusp and apoptotic rate increased along with the increasing concentration of diclofenac sodium (Tab 2).

Tab.2 The cell cycle and apoptotic rate in the three groups

Groups	Distribution of cells cycles(%)			Apoptotic rate (%)
	G_0/G_1 phase	S phase	G_2/M phase	
Control group	74.67	11.43	13.89	2.68
9 μg/ml group	81.42	10.38	8.21	8.95
12.5 μg/ml group	81.34	9.90	8.76	13.61

Discussion

Diclofenac sodium is a kind of nonsteroidal anti-inflammatory drugs (NSAIDs), and it can decrease prostaglandin (PG) level by inhibiting COX to allay fever and ease pain. It has been found that PG can hasten proliferation of cells, so NSAIDs may exert anti-proliferation effect by decreasing PG level. Currently there were many researches on using NSAIDs to prevent and treat tumor[4-5]. Using NSAIDs to suppress neovascularization and prevent posterior capsule opacification (PCO) were also researched[6-8]. Some investigations revealed that NSAIDs can inhibit the proliferation of cancer cells by inducing apoptosis and ceasing cells cycles[9-10]. Our investigation indicated that diclofenac sodium has obvious inhibitory effect on RCECs and showed dosage-effect relation in vitro. The anti-proliferative effect was very apparent on the second day and the inhibitory rates were all above 90% when the final concentration of diclofenac sodium was above 27.27 μg/ml on the third day. These data indicated that the toxicity of diclofenac sodium increased rapidly along with the extending of the action time. It is also reported from ophthalmological clinic that diclofenac sodium could induce cornea melt. In our investigation, the concentrations of diclofenac sodium were lower than that in clinic, but the anti-proliferative effect was very apparent. We think the reason

may be that the vigor of the corneal epithelial cells in vitro is weaker than in vivo and tear dilute the eye drops in vivo. So further studies are required to find the best concentration and acting time of diclofenac sodium.

In this study, flow cytometer was used to observe the effect of diclofenac sodium on inducing apoptosis of corneal epithelial cells in order to discuss the mechanism of its anti-proliferative effect. The observation revealed that diclofenac sodium can induce apoptosis of corneal epithelial cells, and the apoptotic rate increased along with the drug's concentration increase. The results also showed diclofenac sodium changed the cell cycles and increased the cell numbers in G_0/G_1 phase. The apoptotic rates in the experimental groups were higher than that in the control group and typical apoptosis cusp appeared in the experimental groups.

In summary, the study proved that diclofenac sodium had obvious inhibitory effect on RCECs and the possible mechanism of its anti-proliferative effect were inducing cell apoptosis and ceasing cell cycles. So it provided the experimental evidence for using diclofenac sodium to inhibit epithelial ingrowth after LASIK.

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