

Original Article

Expression of IL-2, IL-6, IL-10, and IL-1 β in rats with fungal keratitis

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Abstract: Objective: The objective of this study was to investigate expression of interleukin-2 (IL-2), IL-6, IL-10, and IL-1 β in rats with fungal keratitis. *Methods:* Rat models of fungal keratitis were established *in vitro* and divided into the blank control group, the injury control group and the fungal experimental group. The modeling results were detected via hematoxylin-eosin (HE) staining. The expression levels of IL-2, IL-6, IL-10, and IL-1 β in corneal epithelial tissues were detected via immunohistochemistry at 24 hours after modeling, and the optical density of staining was analyzed. Moreover, relative expression levels of IL-2, IL-6, IL-10, and IL-1 β messenger ribonucleic acid (mRNA) in corneal epithelial tissues were detected via reverse transcription-polymerase chain reaction (RT-PCR) at 4, 8, 16 and 24 hours after modeling. *Results:* At 24 hours after fungal infection, edema occurred in the corneal epithelium and stroma, and there was infiltration of a large number of inflammatory cells in the corneal limbus and stroma. IL-2, IL-6, IL-10, and IL-1 β were hardly expressed in the control group, little expressed in the injury control group and highly expressed in the fungal experimental group. Optical density analyses showed that there were statistically significant differences among the three groups ($P < 0.05$). At 4, 8, 16 and 24 hours after modeling, relative expression levels of IL-2, IL-6, IL-10, and IL-1 β mRNA in corneal epithelial tissues were gradually increased. There were statistically significant differences in relative expression levels of mRNA at each time point between the fungal experimental group and the injury control group ($P < 0.05$), and relative expression levels of mRNA at the same time point also had statistically significant differences between the two groups ($P < 0.05$). *Conclusion:* In this study, the expression of IL-2, IL-6, IL-10, and IL-1 β began to increase gradually after modeling, and they were increased remarkably at 24 hours, suggesting that changes in expression of IL-2, IL-6, IL-10, and IL-1 β may be involved in the occurrence and development of fungal keratitis in rats.

Keywords: IL-2, IL-6, IL-10, IL-1 β , fungal keratitis

Introduction

There have been no antifungal drugs with excellent therapeutic effects yet in clinic, and limited antifungal treatment means are available. Therefore, treatment of fungal keratitis is particularly urgent, which has become one of the great challenges for clinical ophthalmologists [1]. In recent years, the immunology research has attracted the attention of clinical ophthalmologists, hoping to investigate the possible mechanism of fungal keratitis from its clinical pathogenesis and involvement of immune system. At present, it has been found that damage to corneal tissues and defensive immune response in the host are extremely important for the occurrence of fungal keratitis [2, 3]. The

basic function of immune response is for defense, and innate immunity in the body forms an important defensive line of the cornea against fungal infection [4]. Some scholars have found that the combined action of pathogenic bacteria and host can lead to fungal keratitis. After the corneal inflammatory response begins, the immune response will still move on although there are no factors initiating the inflammatory response. Fungi are not always involved in the whole pathogenetic process of the disease, but the patient's immune response participates in the tissue damage process in the late stage of infection [5, 6]. As key anti-inflammatory factors of immune response, interleukin-6 (IL-6) and IL-10 mainly display anti-inflammatory effects in the body, terminating



Figure 1. HE staining ($\times 200$).

the inflammatory response in the body and restraining further damage to tissues [7]. IL-1 β is a kind of cytokine produced in the immune response of the body to infection, which can promote T cells to synthesize and secrete IL-2 during immune response, thus realizing tissue repair [8]. This study aims to observe the expressions of IL-2, IL-6, IL-10, and IL-1 β in rats with fungal keratitis, so as to provide an experimental basis for clinical research.

Materials and methods

Experimental animals

A total of 72 healthy Wistar rats weighing (225 ± 25) g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The experimental animals and conditions met the requirements in the "Management Conditions for the Laboratory Animals" of the State Science and Technology Commission. The animal use conformed to the requirements of Ethics Committee.

Experimental consumables

Rabbit anti-mouse polyclonal antibody (purchased from Biogot Technology Co., Ltd.), immunohistochemistry kit (purchased from Shanghai Chengong Biotechnology Co., Ltd.), diaminobenzidine (DAB) dye (bought from Shanghai LMAI Bio Co., Ltd.) and Prime Script reverse transcription-polymerase chain reaction (RT-PCR) kit (bought from Shanghai Beinuo Biotechnology Co., Ltd.).

Grouping

In immunohistochemical assay, rats with fungal keratitis were divided into the blank control

group ($n=6$), the injury control group ($n=6$) and the fungal experimental group ($n=6$). In RT-PCR, rats were also divided into three groups: the blank control group ($n=6$), the injury control group ($n=24$) and the fungal experimental group ($n=24$).

Establishment of rat model of fungal keratitis

The rat model of fungal keratitis was established according to the method of West DM et al. [9]. In this study, the left eye of rats was used as the experimental eye, while the right eye was used as the injury control eye. Ofloxacin eye drops and Tarivid eye ointment were applied 3 days before operation. Rats were anesthetized intraperitoneally using 10% chloral hydrate, and 0.4% benoxinate hydrochloride was used for corneal surface anesthesia. Epithelial tissues with a diameter of about 4 mm in the central corneas of both eyes were scraped off using a circular blade under an ophthalmic microscope, and the corneal surface was scratched using a 1 mL syringe needle up to the superficial stromal layer. Hypha-stimulating solution was dripped onto the corneal surface and covered the corneal contact lens, and then gentamicin was injected subconjunctivally and the eyelids were sutured. The corneal contact lens were removed at 4 hours after the operation.

Obtaining specimens

At 24 hours after modeling, experimental animals qualified for immunohistochemical assay were anesthetized and executed. At 4, 8, 16 and 24 hours, experimental animals qualified for RT-PCR were anesthetized and executed, and the eyeballs were sterilely removed and fixed in 4% formaldehyde for hematoxylin-eosin (HE) and immunohistochemical staining. Corneal epithelial tissues of rats were scraped off, placed into an EP tube containing cell lysis solution, and stored in a refrigerator at -80°C for RT-PCR.

HE staining

Corneal tissues fixed in formaldehyde were dehydrated for 1 hour, transparentized with xylene for 15 minutes, immersed in wax overnight and embedded. Then tissues were serially sliced into 2 μm -thick sections, followed by dewaxing with xylene, dehydration with gradient alcohol, staining in hematoxylin for 5 min-

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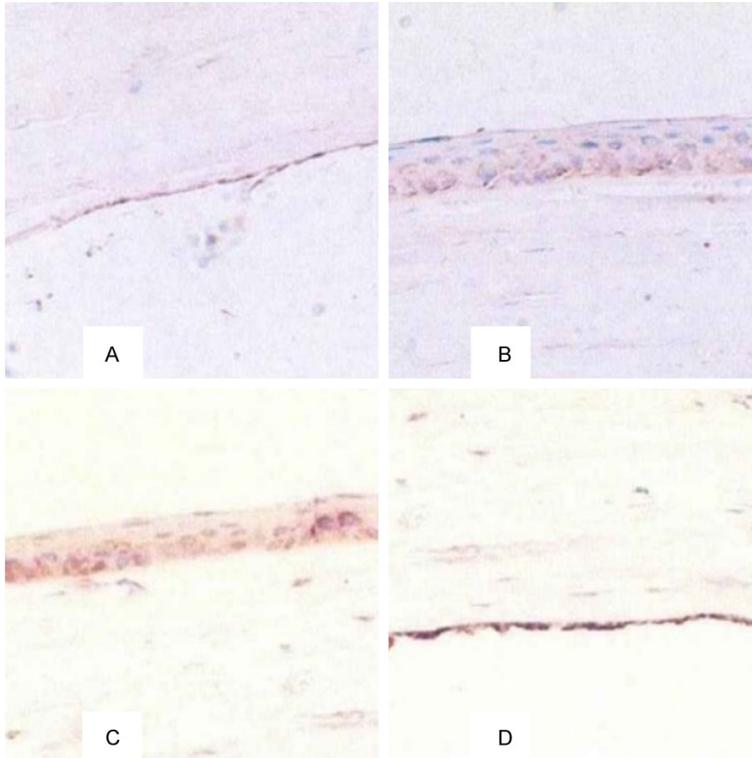


Figure 2. Immunohistochemical staining of corneal epithelial tissues in the blank control group ($\times 200$). A-D: Expressions of IL-2, IL-6, IL-10, and IL-1 β in corneal epithelial tissues.

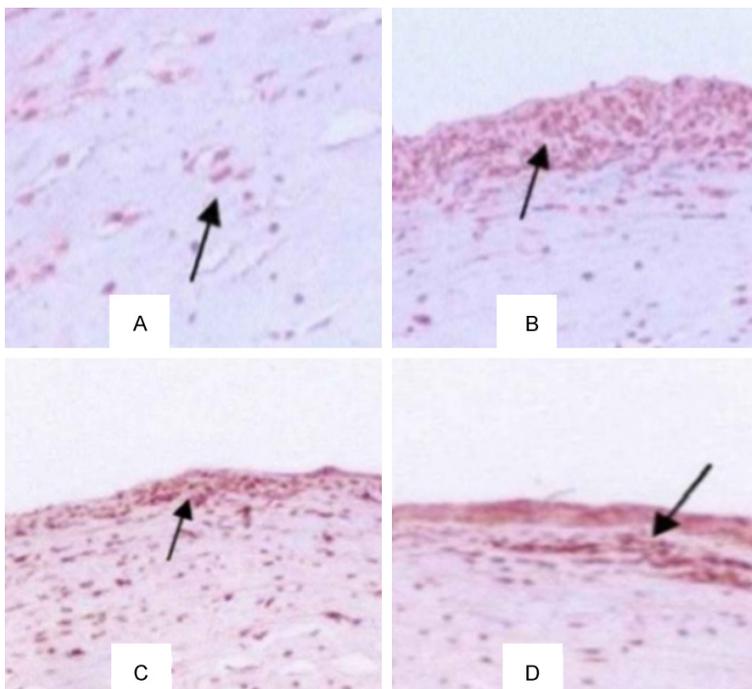


Figure 3. Immunohistochemical staining of corneal epithelial tissues in the fungal experimental group ($\times 200$). A-D: Expression of IL-2, IL-6, IL-10, and IL-1 β in the fungal experimental group.

utes and washing. After differentiation and de-staining, sections were washed with tap water for 1 minute, stained with eosin for 30 seconds and placed into 95% alcohol. Then tissues were dehydrated again with gradient alcohol for 5 minutes, rinsed and sealed using neutral gum, followed by observation under a light microscope.

Immunohistochemical staining

After paraffin sections were dewaxed, hydrated, and rinsed with phosphate buffered saline (PBS), they were blocked for 15 minutes and sealed with 10% serum at room temperature for 15 minutes under non-specific background. Then primary antibodies (MTDH and Wnt5b monoclonal antibodies) were added and placed in the refrigerator at 4°C overnight. After being removed, sections were rinsed with PBS, added with biotin-labeled secondary antibody for incubation at room temperature for 30 minutes and rinsed again with PBS. Then sections were incubated with streptavidin-peroxidase solution at room temperature for 30 minutes and washed with PBS, followed by color development via DAB, washing with tap water, counterstaining with hematoxylin, de-staining, and transparency. Finally, sections were sealed with neutral gum and observed under the microscope.

Evaluation of results: Yellow particles in the tissues were used as positive criteria. The optical density of IL-2, IL-6, IL-10, and IL-1 β staining was analyzed using the VIDAS-21

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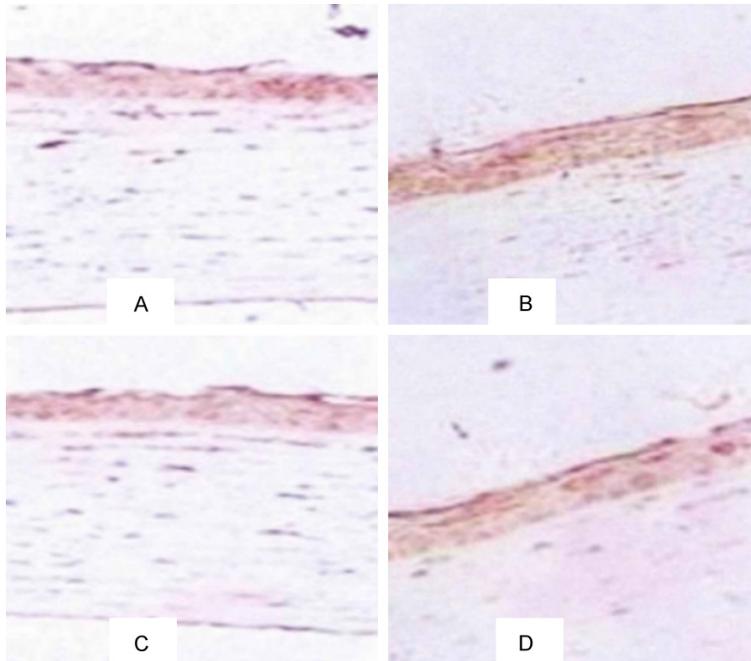


Figure 4. Immunohistochemical staining of corneal epithelial tissues in the injury control group ($\times 200$). A-D: Expressions of IL-2, IL-6, IL-10, and IL-1 β in the injury control group.

computer color image analysis system, and 5 fields of view were randomly selected in each specimen.

RT-PCR

The total ribonucleic acid (RNA) was extracted from fungal keratitis tissues according to instructions of the TRIGene kit, its concentration and purity were determined using a spectrophotometer, and the absorbance (A_{260}/A_{280}) value was between 1.8 and 2.0. Primer sequences were synthesized by Shanghai Jiran Biotechnology Co., Ltd., according to instructions of the Reverted First Strand cDNA Synthesis Kit (Thermo, K1622). IL-2: forward primer: 5'-ATGTACAGGATGCAACTCCTG-3', reverse primer: 5'-TCAAGTCAGTGTGAGATGATGCTT-3'. IL-6: forward primer: 5'-AGCCACTGCCTCCCTACTT-3', reverse primer: 5'-GCCATTGCACAACTCTTTTCTC-3'. IL-10: forward primer: 5'-TGCCTTCAGTCAAGTGAAGAC-3', reverse primer: 5'-AAACTCATTATGGCCTTGTA-3'. IL-1 β : forward primer: 5'-CCTCGTGTGTCGGACCCATA-3', reverse primer: 5'-CAGGCTTGCTCTGCTTGTGA-3'. GAPDH: forward primer: 5'-CAGGAGGCA-TTGCTGATGAT-3', reverse primer: 5'-GAAGGC-TGGGGCTCATTT-3'. RNA was reversely tran-

scribed into cDNA via RT-PCR according to instructions of the real-time fluorescence quantitative PCR kit ($2 \times$ RealStar Green Power Mixture, GenStar, A311). The reaction system was a total of 20 μ L in volume, and reaction conditions are as follows: 95 $^{\circ}$ C for 10 minutes, 95 $^{\circ}$ C for 30 seconds, 59.4 $^{\circ}$ C for 30 seconds, a total of 40 cycles, 95 $^{\circ}$ C for 15 seconds, and then cooling to 65 $^{\circ}$ C.

Agarose gel electrophoresis and analysis

After 5 μ L products in the tube after RT-PCR were taken, 1 μ L loading buffer was added, followed by electrophoresis under 100 mV for 40 minutes, and then observation and photography of gel electrophoresis bands using an ultraviolet imaging system. Analysis of

gray levels was performed using the Gel-Pro analyzer. The integral absorbance values of IL-2, IL-6, IL-10, IL-1 β and GAPDH as an internal reference were detected, respectively, and the relative integral absorbance values of IL-2, IL-6, IL-10, and IL-1 β were calculated as the relative expression level.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 19.0 software was used for data analysis. Experimental data are presented as mean \pm standard deviation, the one-way analysis of variance was used for the overall comparison, and least significant difference (LSD) test was adopted for the pairwise comparison among groups. $\alpha=0.05$ was used as the inspection statistical level.

Results

HE staining results

At 24 hours after fungal infection, it could be seen under the light microscope that edema occurred in the corneal epithelium and stroma, and there was infiltration of a large number of

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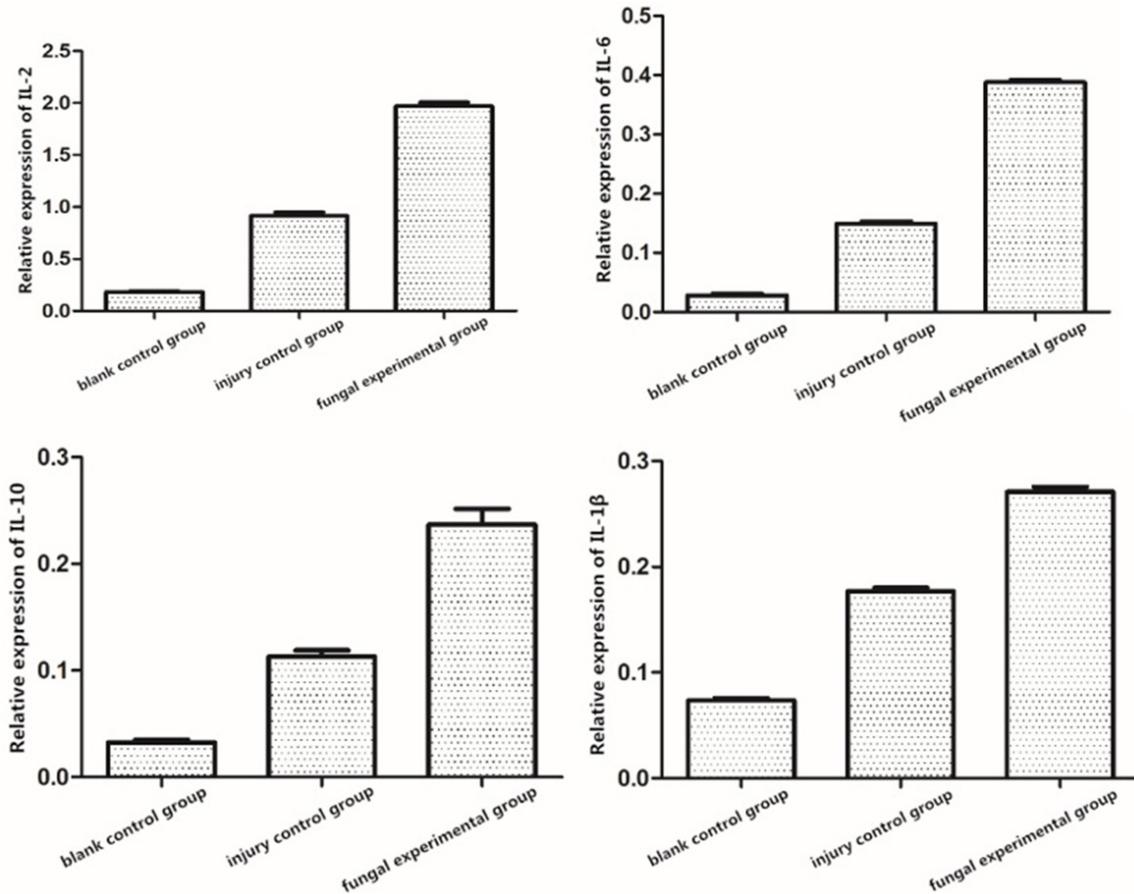


Figure 5. Comparison of average absorbance value in immunohistochemical staining. Detection of relative mRNA expression in corneal epithelial tissues via RT-PCR.

inflammatory cells in the corneal limbus and stroma (**Figure 1**).

Immunohistochemical staining results

Results of immunohistochemical staining revealed that brown-yellow particles in cells indicated the positive expression, and the darker the color, the stronger the positive expression. At 24 hours after modeling, IL-2, IL-6, IL-10, and IL-1 β were not expressed in corneal epithelial tissues in the blank control group (**Figure 2**).

IL-2, IL-6, IL-10, and IL-1 β were mainly expressed in the epithelial layer and stromal layer, which were highly expressed in corneal epithelial tissues in the fungal experimental group (**Figure 3**).

Expression levels of IL-2, IL-6, IL-10, and IL-1 β in the injury control group were significantly lower than those in the fungal experimental group (**Figure 4**).

Comparison of absorbance value in immunohistochemical staining

The absorbance values of IL-2, IL-6, IL-10, and IL-1 β were lowest in the blank control group, moderate in the injury control group and highest in the fungal experimental group. There were statistically significant differences among the three groups ($P < 0.05$), and the pairwise comparison also displayed a statistically significant difference ($P < 0.05$) (**Figure 5**).

At 4, 8, 16, and 24 hours after modeling, the relative expression levels of IL-2, IL-6, IL-10, and IL-1 β mRNA in corneal epithelial tissues were gradually increased. There were statistically significant differences in relative expression levels of mRNA at each time point between the fungal experimental group and the injury control group ($P < 0.05$), and relative expression levels of mRNA at the same time point also had statistically significant differences between the two groups ($P < 0.05$) (**Figure 6**).

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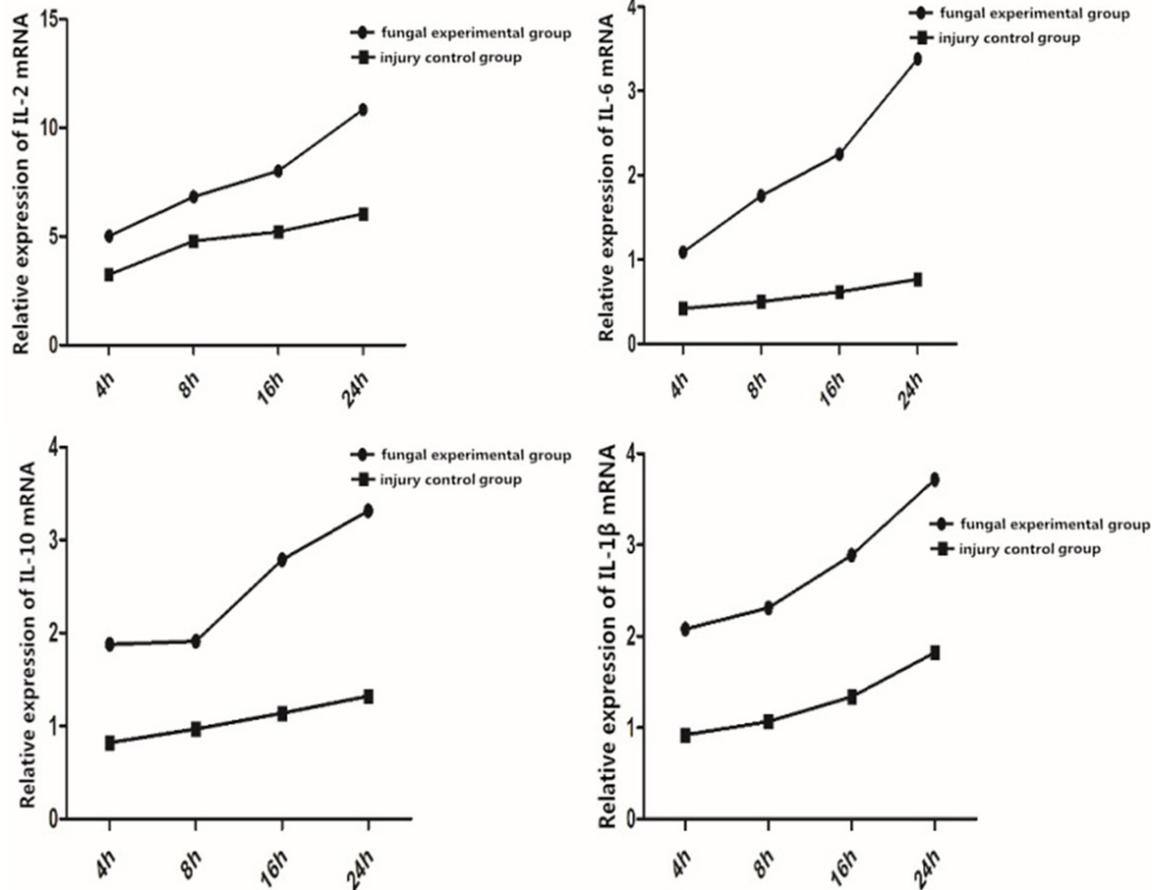


Figure 6. Detection of relative mRNA expression in corneal epithelial tissues via RT-PCR.

Discussion

Fungal keratitis is an extremely serious infectious corneal inflammatory disease. There are very few drugs for fungal diseases and insufficient corneal donors, so that the blindness rate in patients with fungal keratitis is extremely high [10]. At present, more than one hundred kinds of fungi have been found to lead to eye infection, ultimately causing eye diseases. In this study, a rat model was established to simulate the process of human corneal fungal infection, and expression of IL-2, IL-6, IL-10, and IL-1 β in fungal corneal epithelial tissues were detected, so as to understand the pathogenesis of fungal keratitis more deeply.

In this study, many inflammatory cells and inflammatory response processes were observed in corneal epithelial tissues with fungal infection, and more polymorphonuclear leukocytes were found in the later stage of disease, which is consistent with the report of Bryant-

Hudson KM [11]. Immunological studies have shown that specific immune response occurs rapidly, and there was infiltration of a large number of inflammatory cells in the early stage of immune response in the body. In the mid-late stage of inflammatory response, T lymphocytes began to exert a proliferative effect and gradually dominate in inflammatory response [12, 13]. According to the mainstream view currently, the cellular immune response always dominates in anti-fungal infections, and T helper (Th) cells are involved in the entire immune response [14]. Th1 cells can enhance the body's anti-fungal ability, and result in the secretion of pro-inflammatory factors (such as IL-1 β and IL-2), thereby killing pathogens, which are closely correlated with the protective inflammatory response. Th2 cells can produce anti-inflammatory cytokines (IL-6, IL-10, etc.), thereby reducing excessive damage, which is correlated with inhibiting immune inflammatory response [15]. Th1 and Th2 are in a dynamic

equilibrium and regulated by a variety of factors.

Some scholars have found that IL-6 plays an extremely important defensive role in anti-fungal infection [16]. This study demonstrated that IL-6 was hardly expressed in the blank control group, but began to increase in the fungal experimental group and reached the peak at 24 hours after modeling, proving that IL-6 expression in corneal epithelial tissues coincides with the degree of local inflammatory response, and suggests that IL-6 may exert its effect as a protective cytokine. IL-10 is an effective anti-inflammatory cytokine, and it can inhibit the secretion of IFN and the protective Th1-type cell response through inhibiting cells from secreting other types of interleukins [17]. This study reveals that there is a certain correlation between IL-10 expression and the occurrence of fungal infection. IL-10 expression was not expressed in blank control group, gradually increased after infection and reached the peak at 24 hours. IL-10 expression may thus be increased in the body to resist the Th1-type inflammatory response, which is similar to the research conclusion of Fabiani C [18]. Moreover, IL-1 β can bind to the receptors of immunoglobulin superfamily to produce cytokines in immune response and effectively promote T cells to produce IL-2 and other cytokines, thereby realizing tissue repair in immune response [19, 20]. With constant extension of infection time in this study, the content of both IL-1 β and IL-2 was the highest at 24 hours, suggesting a synergistic increase.

In conclusion, the expression of IL-2, IL-6, IL-10, and IL-1 β increases gradually after modeling in this study, and remarkably at 24 hours, suggesting that changes in expression of IL-2, IL-6, IL-10, and IL-1 β may be involved in the occurrence and development of fungal keratitis in rats.

Disclosure of conflict of interest

None.

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