

Changes in corneal innervation and pain responses in fungal keratitis

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INTRODUCTION

Fungal keratitis (FK) is a popular eye disease worldwide, especially in developing countries dominated by agricultural populations^[1]. Recently, the incidence of this disease has elevated dramatically^[2]. In the clinic, FK patients often present with decreased corneal pain response, which causes patients to lose opportunities for early treatment and causes substantial vision damage that produces a need for corneal transplantation. The lack of pain responses highlights the complex mechanisms underlying FK symptoms and the difficulty in alleviating them. The reason for pain insensitivity in FK has not been clarified; however, such clarification is important not only to enable timely and effective treatment of FK patients but also to further understand the pathogenic mechanisms of FK.

The cornea is the most densely innervated structure in the human body^[3]. Corneal nerves not only play essential roles in eye sensations of pain, touch and temperature but also have effect on blink reflexes, wound healing, and tear secretion^[4]. Sensory nerve endings of the corneal surface are defined as nociceptors. Eye pain is mediated by nociceptors of the trigeminal nerve terminals located on corneas^[5]. In addition to the conduction of pain, peripheral nerves also contain endogenous analgesic protein receptors. β -endorphin (β -EP) is an endogenous opioid peptide in peripheral tissue that can play an important role in pain control^[6-7]. β -EP has strong affinity for both μ and δ receptors, and it mainly acts on μ receptors to produce its effects. Opioid receptors produce antinociceptive or analgesic effects^[8]. The number of peripheral opioid receptors has been reported to increase in the inflammatory state^[9]. In acute inflammation, opioid peptides are almost exclusively expressed by immune cells^[10-11]. After the opioid peptide is released, it acts on the corresponding receptors of peripheral sensory nerve endings to trigger antinociception^[12]. This

Abstract

• **AIM:** To characterize changes in the cornea nerve and pain responses in fungal keratitis (FK).

• **METHODS:** A retrospective analysis of *in vivo* confocal microscopy images of 11 FK corneas was performed, and the results were compared with those for 11 normal corneas. Subbasal corneal nerves were analyzed for total nerve number, main nerve trunk number, branching patterns and tortuosity. C57BL/6 mice were infected with *Aspergillus fumigatus*. Disease severity was determined through clinical scoring and slit lamp photography. Corneas were harvested at 1, 3, 5, and 7d post infection (p.i.) and assessed for β III tubulin. Corneal mechanical sensitivity thresholds were detected by von Frey test. β -endorphin (β -EP) and μ receptor protein expression was detected through Western blotting.

• **RESULTS:** Total nerve number, main nerve trunk number, and nerve branching were significantly lower in FK patients than in controls, but tortuosity was not significantly different. In infected mice, subbasal nerve density decreased from 1d p.i., reaching a minimum at 5d p.i. Clinical scores rose at 1d p.i., peaked at 3d p.i., and decreased at 5d p.i. Mechanical sensitivity thresholds showed the same trends. β -EP and μ receptor protein expression increased after infection.

• **CONCLUSION:** Corneal nerve density is lower in FK patients and *Aspergillus fumigatus*-infected mice than in controls. Pain sensitivity decreases with postinfection corneal ulcer aggravation. β -EP and μ receptor proteins are both upregulated in infected mouse corneas.

• **KEYWORDS:** keratitis; pain; fungal; innervation; subbasal nerve; mice

mechanism has been proven in inflammation of surrounding tissues such as the skin^[13-14], but the expression of these molecules has not yet been studied in FK.

It is important to elucidate the reason underlying the lack of pain response in FK. In this study, we focused on changes in corneal nerves and pain responses in FK. We show that corneal nerve density was lower after *Aspergillus fumigatus* (*A. fumigatus*) infection. Pain sensitivity decreased with postinfection corneal ulcer aggravation. β -EP and μ receptor proteins were both upregulated in infected mouse corneas.

MATERIALS AND METHODS

Ethical Approval All patients were informed of the purpose of the study and their consent was obtained in accordance with the Declaration of Helsinki. All mice were treated in a humane way according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research.

In Vivo Confocal Microscopy A retrospective review of patients with FK in the Affiliated Hospital of Qingdao University between January 2013 and November 2018 was carried out. By obtaining positive culture results from microbiological laboratory data, and searching the confocal microscope results of Confoscan 4 slit-scanning confocal microscope (Nidek Co. Ltd., Japan), found cases of filamentous hyphae positive in the cases. Exclusion criteria for FK patients included wearing contact lens, past history of infectious keratitis, ocular inflammatory disease or eye trauma; ophthalmic surgery for the first three months; or diabetes. Examination of all patients was performed by the same ophthalmologist. The affected eye was anesthetized using 0.5% proparacaine eye drops, and the head and eyes were fixed in front of the microscope. The examiner applied an appropriate amount of gel to the lens and adjusted the handle on the main unit to bring the gel on the lens into contact with the cornea. Images were saved for data analysis. For each subject, three high-quality subbasal nerve bundle images were selected for analysis. For image analysis, we referred to the criteria and methods used by Kurbanyan *et al*^[15]. Image J was used to analyze and calculate all parameters retrospectively by a blinded observer.

Animals and Corneal Infection Jinan Pengyue Experimental Animal Co. Ltd. (Jinan, China) provided C57BL/6 mice (female, 8wk) without specific pathogens. The corneas were examined distinctly with slit lamp microscope before use in experiments. To anesthetize mice, 8% chloral hydrate (0.08 mL/mouse) was applied *via* intraperitoneal injection. Mice corneas were scraped to form a wound (2 mm in diameter) in the central epithelium under stereomicroscope, and then treated with 5 μ L of *Aspergillus fumigatus* aliquot consistent with previously described method^[16]. Suturing eyelids after covering cornea surface with soft contact lens to prevent further fungi leakage.

Immunohistochemistry After cervical dislocation of the mice, mouse eyeballs were removed and fixed with 1.3% paraformaldehyde in phosphate buffer saline (PBS) at room temperature for 1h. Then, the corneas were dissected, and radial incisions were made to ensure that the corneal tissues could be flat-mounted. The corneas were then washed in PBS five times for five minutes per wash, then 1% Triton X-100 in PBS was used to permeabilize corneas at room temperature for 60min, and 20% sputum serum in blocking buffer was used to block them for 1h. The corneas were then incubated in 125 μ L of a cocktail of primary antibodies against β III (3 μ g/mL; Abcam, Cambridge, UK) for 2h at room temperature followed by an incubation overnight at 4°C. The corneas were incubated in anti-rabbit secondary antibody (1:200; Cwbiochem, Wuhan, China) and DAPI (1:10, Solarbio) for 2h. After washes, the corneas were fixed on slides. Images were captured with a fluorescence confocal microscope.

Von Frey Test To examine the pain response after infection, a behavioral test was performed. The von Frey test was employed to examine corneal mechanical sensitivity thresholds^[17]. The mice were wrapped in surgical towels beneath a stereoscopic microscope and gently held by hand to ensure that the eye was completely exposed. A set of calibrated von Frey hairs (Stoelting Co., IL, USA) was used to probe the areas surrounding the ulcer of the cornea. Blink response was assessed in untreated controls and mice infected with *A. fumigatus* ($n=5$ per group); a positive response for the test was recorded when a mouse exhibited a blink response. Each cornea was mechanically stimulated five times with the von Frey hairs (0.008, 0.02, 0.04, 0.07, 0.16 and 0.40 g) to assess the pain response.

Western Blot Analysis Proteins were isolated from corneas using RIPA buffer (Solarbio, Beijing, China) mixed with phenylmethanesulfonyl fluoride (PMSF) (100:1, Solarbio) for 2h. The concentration of total proteins was evaluated with BCA Protein Assay Reagent (Solarbio). Western blotting system was established and performed as previously described^[2]. Primary antibodies against pomc (1:3000; Elabscience, Wuhan, China), μ receptor protein (1:1000; Abcam, Cambridge, UK) and GAPDH (1:3000; R&D, Minneapolis, MN) were applied.

Statistics Statistical analyses of the differences between the two groups were performed using two-tailed Student's *t*-tests in GraphPad Prism 5.0. The relationships between the clinical scores and the von Frey hair forces were assessed by Spearman rank correlation analysis. All data are expressed as the mean \pm standard deviation (SD). $P<0.05$ was considered statistically significant.

RESULTS

In Vivo Confocal Microscopy Analysis in Fungal Keratitis Patients Diagnoses of all patients were confirmed by finding

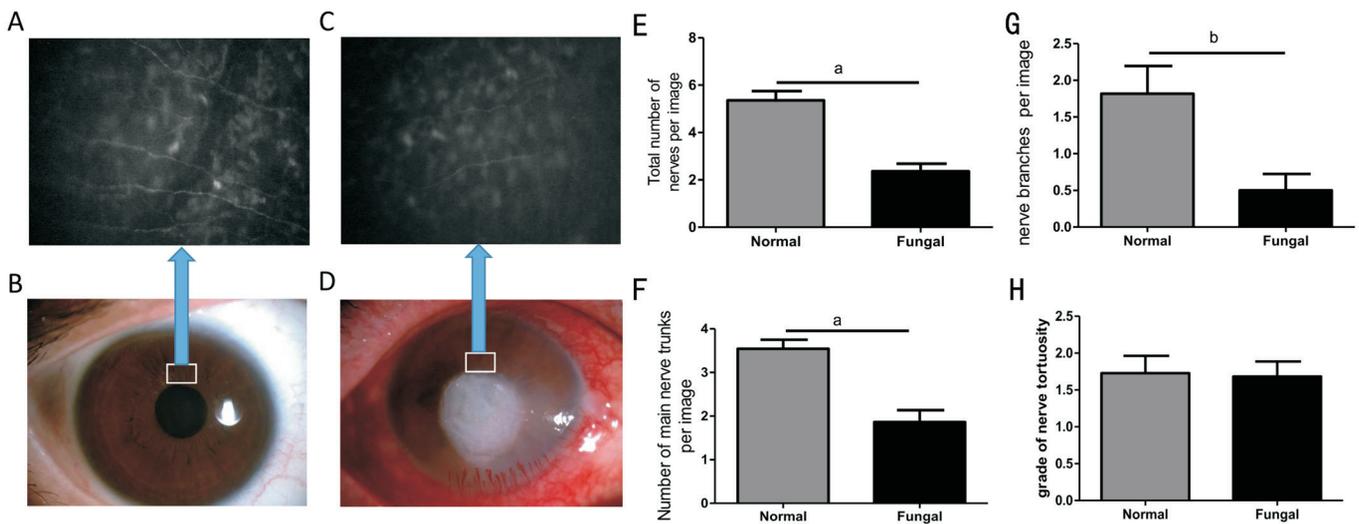


Figure 1 Representative the slit-lamp photographs and *in vivo* confocal microscopy (IVCM) images of normal cornea and FK patients IVCM analysis showing a reduced total nerve count in FK patients (C) compared with normal corneas (A). D and B are the slit-lamp photographs of FK patient and normal cornea respectively. The total nerve counts were significantly lower in patients with FK than in normal controls (E). The average number of main nerve trunks was also significantly lower in the FK group than in the normal control group (F). Nerve branching was found to be diminished in FK corneas compared to normal corneas (G). However, tortuosity was not significantly different between the FK group and the normal group (H). ^aP<0.001, ^bP<0.01.

out fungal hyphae on *in vivo* confocal microscopy (IVCM) or by positive culture results in microbiology laboratory analysis. Among the 11 FK patients, 6 were infected with *Fusarium* and 5 were infected with *Aspergillus*, and the patients had suffered from corneal ulcers for 12±4d. The clinical results are summarized in Table 1.

The discovered nerves were noted to have quantitative and morphological changes in FK patients (Figure 1C, 1D) compared to normal controls (Figure 1A, 1B). The total nerve counts were significantly lower in patients with FK (2.4±1.0) than in normal controls (5.4±1.2, P<0.001; Figure 1E). The average number of main nerve trunks was also significantly lower in the FK group (1.9±0.9) than in the normal control group (3.5±0.7, P<0.001; Figure 1F). Nerve branching was found to be diminished in FK corneas (0.5±0.7) compared to normal corneas (1.8±1.2, P<0.01; Figure 1G). However, the tortuosity of the corneal nerve was not significantly different between the FK group (1.7±0.6) and the normal group (1.8±0.7; Figure 1H).

Clinical Scores and Von Frey Test Results in C57BL/6 Mice

To illustrate the disease response in infected mice, we used a slit lamp to take photographs at 1, 3, 5, and 7d post infection (p.i.; Figure 2A). The clinical score (Figure 2B) increased from 1d p.i. (P<0.001), peaked at 3d p.i. (P<0.001), declined by 5d p.i. (P<0.001) and was reduced to the lowest level at 7d p.i. (P<0.001). We further used von Frey hairs to explore changes in mechanical sensitivity thresholds in infected mouse corneas. We touched the mouse corneas with different types of filaments and recorded the type of filament that elicited a blink

Table 1 Demographic data of patients with fungal keratitis in comparison with normal controls

Parameters	No. of patients (n)	Age (y)	Gender (M/F)	Days of infection (d)	mean±SD
Normal	11	40±16	6/5	-	
Fungal	11	37±10	5/6	12±4	

Days of infection denotes the time elapsed since diagnosis of the infection until the IVCM or fungal scraping was performed.

response. In the mouse model of *A. fumigatus* keratitis, the von Frey filament force (Figure 2C) was increased at 1d p.i. (P<0.01) and peaked at 3d p.i. (P<0.001). Moreover, there was a strong positive correlation between clinical score and von Frey filament force (Figure 2D, P<0.001), suggesting that the cornea became less pain sensitive with increasing severity of ulceration.

Changes in Corneal Nerves in Infected C57BL/6 Mice

To research the effect of *A. fumigatus* infection on corneal nerve structure and to examine the relationship between structure and pain insensitivity, C57BL/6 mice corneas were harvested to take immunohistochemical (IHC) staining with an β III tubulin antibody, which against the panneuronal marker, at 1, 3, 5, and 7d p.i. Under the microscope, we found that the nerve structure could not be clearly observed in the central ulcer area of the cornea after *A. fumigatus* infection, possibly due to severe corneal edema caused by the infection. In addition, the nerve could not be stained normally. We thus observed and imaged the areas surrounding the ulcer. As we can see in the corneal whole-mount images (Figure 3A-3E), infection caused progressive nerve loss starting at 1d p.i. (P<0.001), with more

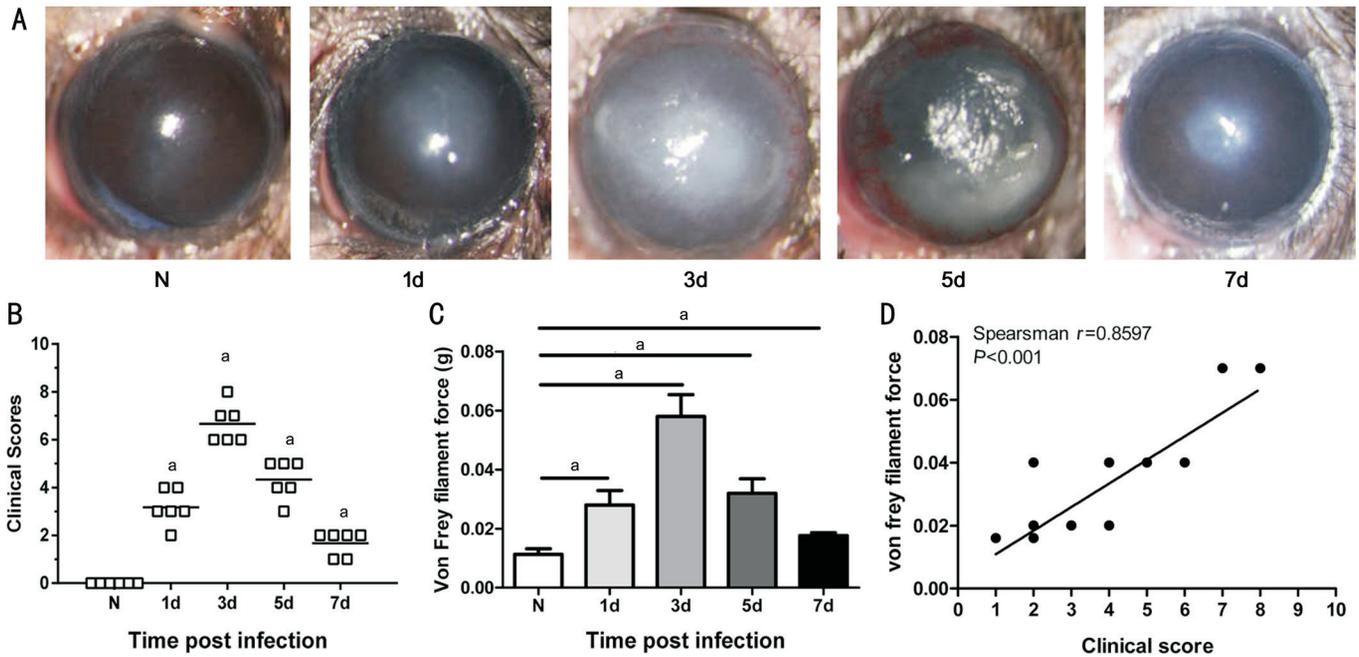


Figure 2 Clinical score and Von Frey test in C57BL/6 mice Take photographs at 1, 3, 5, and 7d p.i. to illustrate the disease response in infected mice (A). The clinical score (B) increased from 1d p.i., peaked at 3d p.i., declined by 5d p.i. and was reduced to the lowest level at 7d p.i. The von Frey filament force (C) was increased at 1d p.i. ($P<0.01$) and peaked at 3d p.i. There was a strong positive correlation between clinical scores and force of Von Frey filament (D). $^aP<0.001$.

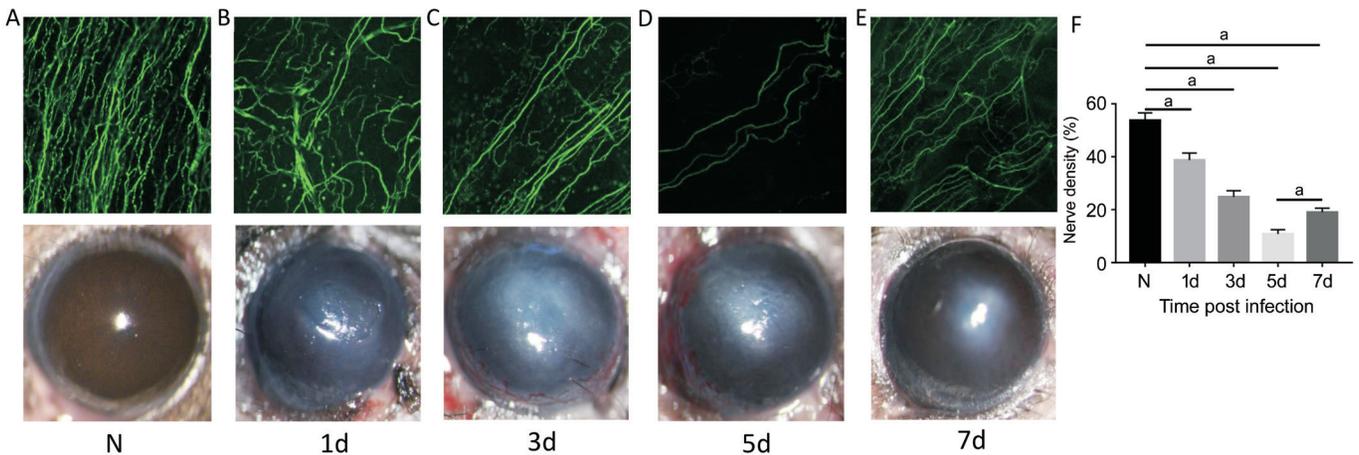


Figure 3 Changes of corneal nerves in infected C57BL/6 mice In comparison to the normal group (A), progressive nerve loss starting at 1d p.i. (B), with more pronounced loss at 3d p.i. (C) and 5d p.i. (D). The number of nerves at 7d p.i. (E), was higher than that at 5d p.i. Magnification (A-E): 200 \times . Quantification of fluorescence integrated density of β III tubulin using Image J (F). $^aP<0.001$.

pronounced loss at 5d p.i. ($P<0.001$). The number of nerves at 7d p.i., was higher than that at 5d p.i. ($P<0.001$).

Expression of β -EP and the μ Receptor in Infected C57BL/6 Mice To further study the causes of pain insensitivity in FK, we tested the protein levels of β -EP and the μ receptor in normal (uninfected) and infected C57BL/6 corneas by Western blot analysis. The results indicated that β -EP protein levels (Figure 4A, 4B) were upregulated in infected mouse corneas compared with normal corneas at 1, 3 and 5d p.i. (all $P<0.001$), peaked at 3d p.i. At the same time, μ receptor protein (Figure 4C, 4D) was also upregulated at 1, 3 and 5d p.i. (all $P<0.001$) in the infected group compared with the normal group.

DISCUSSION

FK is a severe infective corneal disease caused by pathogenic fungi that is accompanied by high rates of blindness^[18]. However, in the clinic, treatment is delayed in many FK patients due to a lack of severe pain, resulting in corneal perforation and even loss of vision. In the current study, FK patients who had been infected for 12 \pm 4d presented with mild pain, confirming the lack of pain responses in FK. (It would be better if the Cochet-Bonnet esthesiometry test was used to evaluate corneal sensation in patients, but since our research is retrospective and esthesiometry is not our routine examination, we did not perform it.)

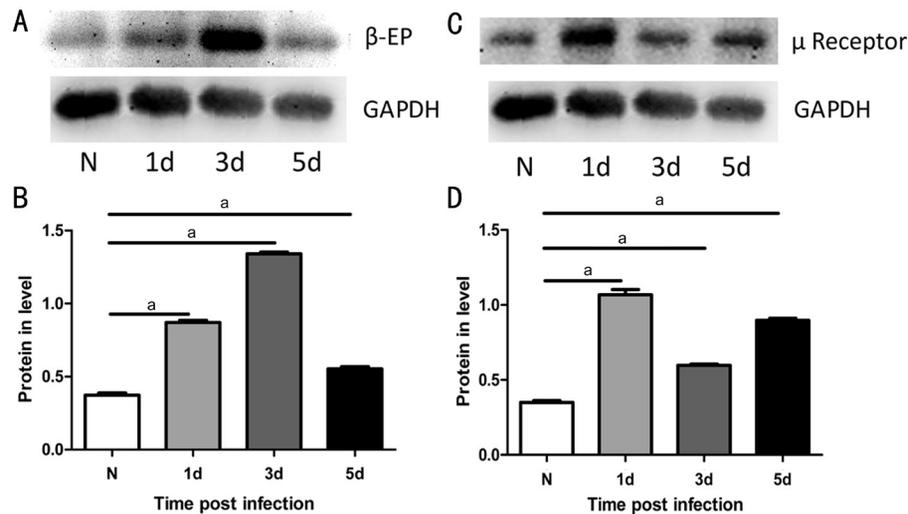


Figure 4 Expression of β -EP and μ receptor in infected C57BL/6 mice β -EP protein levels (A, B) were upregulated in infected mouse corneas compared with normal corneas at 1, 3 and 5d p.i., peaked at 3d p.i. At the same time, μ receptor protein (C, D) was also upregulated at 1, 3 and 5d p.i. in the infected group compared with the normal group. ^a $P < 0.001$.

The cornea is mainly dominated by sensitive fibers which originated from the eye area of the trigeminal ganglion^[3]. Eye pain is caused by nociceptors expressed on the nerve endings of the trigeminal neurons that innervate the surface of the eye. Chucair-Elliott *et al*^[19] found that the loss of corneal sensation in herpes stromal keratitis is related to decreases in corneal sensory nerves. In our study, we found that the total number of nerves, average number of main nerve trunks and degree of nerve branching were all lower in FK patients than in normal subjects. These findings are consistent with previous studies on the corneal nerves of FK patients^[15].

To further explore the change in corneal nerves and pain responses, we established an *A. fumigatus* mouse model. For the first time, we found that the number of subbasal corneal nerves was decreased in the infection group, with the lowest level at 5d p.i. To evaluate topical resiniferatoxin (RTX) as a corneal analgesic, Bates *et al*^[17] performed von Frey tests to analyze mechanical sensitivity thresholds in corneas. To evaluate pain responses after *A. fumigatus* infection, we similarly performed von Frey tests at various time points after infection. In our research, the clinical score was highest at 3d p.i., but the force of corneal touch needed to elicit a blinking response was also the greatest at this time point. Moreover, we also found that corneas became less sensitive to pain with increasing severity of ulceration. The results showed that there was a significant correlation between the clinical score and the von Frey hair force needed to elicit a response. The results also indicated that with increasing clinical scores, corneal subbasal nerve density decreased and the von Frey hair force needed to elicit a blink response increased. We noticed that corneas had the least sensitivity at 3d p.i.; however, the number of subbasal nerves at 5d p.i. was fewer than 3d p.i. We speculate that

the recovery of pain at 5d p.i. may be due to the reduction of inflammatory cells, causing the release of analgesic substances to decrease.

There were endogenous analgesic protein receptors in the peripheral nerves. β -EP is a major member of the endogenous analgesic system. When inflammation occurs, inflammatory cells release β -EP, which then acts on receptors on the peripheral nerves to inactivate ion channels and hyperpolarize the nerves, thereby exerting an analgesic effect^[20-21]. To explore whether β -EP and its μ receptor are expressed in *A. fumigatus* keratitis, we detected their protein levels for the first time. The results showed that both β -EP and μ receptor protein levels were significantly elevated by *A. fumigatus* infection. β -EP protein expression was upregulated in infected mouse corneas compared with normal corneas from 1d p.i. and peaked at 3d p.i. At the same time, μ receptor protein expression was also upregulated at 1d p.i., 3d p.i. and 5d p.i. compared with the normal group. A previous study reported that P-enkephalin (P-ENK, a precursor of the endogenous opioid enkephalin) mRNA levels were significantly decreased in patients with dry eyes who had significant pain^[22]. In addition, another study^[23] demonstrated that treatment with an anti- β -EP antibody reduced endogenous antinociceptive activity in rats infected with unilateral hindpaw inflammation induced by Freund's adjuvant. Our findings are consistent with studies showing that the numbers of opioid receptors in the periphery are increased during inflammation^[9].

In summary, the data presented here indicated that both FK patients and C57BL/6 mice infected with *A. fumigatus* had profound reductions in subbasal corneal nerves compared with normal controls. In infected mouse models, increased ulcer severity was associated with reduced pain sensitivity.

Furthermore, β -EP and its μ receptor were both upregulated after infection. Extensive studies are needed to investigate the specific mechanisms by which nerve degeneration and endogenous analgesic protein activity mediate pain insensitivity in FK.

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