• Basic Research •

Anti-inflammatory effects of astaxanthin against fungal keratitis

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Abstract

● **AIM:** To characterize effect of astaxanthin (ASX) in *Aspergillus fumigatus* (*A. fumigatus*) induced keratitis in mouse model.

● **METHODS:** *In vivo*, fungal keratitis mouse model was established in C57BL/6 mice using *A. fumigatus*, followed by ASX or dimethyl sulfoxide (DMSO) treatment. Clinical responses were evaluated by clinical score and myeloperoxidase (MPO) assay. Inflammatory cytokines were assessed by reverse-transcription polymerase chain reaction (RT-PCR), Western blot, immunofluorescence, and enzyme-linked immuno sorbent assay (ELISA).

● **RESULTS:** In animal model, ASX improved corneal transparency and clinical response, suppressed the expression of inflammatory cytokine like IL-1β, TNF-α, and HMGB-1. Neutrophil levels have been shown to decrease in ASX-treated cornea by immunofluorescence and MPO. TLR2 and TLR4 levels were lower in ASX-treated group than DMSO-treated.

● **CONCLUSION:** ASX can suppress inflammatory response and reduce inflammatory cytokine production in mice model with *A. fumigatus* keratitis.

● **KEYWORDS:** astaxanthin; fungal keratitis; *Aspergillus fumigatus*; anti-inflammation; neutrophil
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INTRODUCTION

Fungal keratitis (FK) is a common causes of infected corneal diseases in developing countries[1-2], *Aspergillus fumigatus* (*A. fumigatus*) and *Fusarium solani* are most common pathogens in FK[3-4]. Compared with viral or bacterial corneal ulcers, fungal corneal ulcers tend to have a worse prognosis because of the uncontrollable innate immune response and disadvantages of conventional medications such as poor permeability, cytotoxicity, and drug-resistance[5-6].

Astaxanthin (ASX) is a ketocarotenoid first extracted from a lobster. It belongs to terpenes and can be isolated from halobios[7-8]. To date, ASX has a variety of biological functions, including antioxidant, anti-inflammatory, anti-apoptotic, neuroprotective and anti-tumor properties[9-10]. Recently ASX was also used in aquaculture as a dietary supplement which promote the growth and reproductive performance of fish, and also booste immune system in fish and shellfish[11-12].

More importantly, a therapeutic effect of ASX on mice mastitis model was reported by Dolma *et al*[13]. In addition, it demonstrates that ASX inhibits the inflammatory response in pathogenesis of uveitis[14]. Singh *et al*[10] reported that ASX can effectively reduce inflammatory factors in the process of dermatitis, alleviate the severity of the disease in skin. These results suggest that ASX can inhibit inflammation.

As the first line of immune system, innate immunity recognizes and eliminates fungi through pattern recognition receptors (PRRs), which recognizes pathogen-associated molecular patterns (PAMPs) in pathogens and promotes the secretion of inflammatory factors[15]. Toll-like receptors (TLRs) are important PRRs involved in FK, in which TLR2 and TLR4 are the main PRRs expressed in corneal epithelial cells[16-17].

However, protective effects of ASX in FK is still not established. In order to explore the effect of ASX in *A. fumigatus* induced keratitis. We established an *A. fumigatus* mouse model, set different groups and give corresponding treatment. In order to explore the clinical inflammatory level of cornea, analyze the number of neutrophils and myeloperoxidase (MPO) activity, inflammatory factors (IL-1β, TNF-α, and HMGB1), TLR2, and TLR4 in cornea.

MATERIALS AND METHODS

Ethical Approval Mice were treated in accordance with the

Statement for the Use of Animals in Ophthalmic and Vision Research by the Association for Research in Vision and Ophthalmology (ARVO).

Reagents We obtained ASX from Sigma-Aldrich Chemical Co. RNAiso Plus, TB Green™ Premix Ex Taq™ II and reverse-transcription polymerase chain reaction (RT-PCR) kit were from TaKaRa (Dalian, Liaoning Province, China). Phosphate buffer saline (PBS), Bicin-choninic acid (BCA) protein assay kit, phenylmethylsulfonyl fluoride (PMSF) were derived from Solarbio (Beijing, China). TNF-α, IL-1β and HMGB-1 enzyme-linked immuno sorbent assay (ELISA) kits were from Elabscience. Antibodies of β-actin, TLR2 and TLR4 for Western blot were from *Abcam* (Cambridge, UK). TLR2 and TLR4 antibody for immunofluorescence stain was from Abclonal (Wuhan, China). Rabbit anti-mouse neutrophil antibody, fluorescein isothiocyanate (FITC) labeled donkey anti-rabbit secondary antibody, 4',6-diamidino-2-phenylindole (DAPI) solution for immunofluorescence stain was from CST (Cell Signaling Technology, USA).

Culture of Primary *A. fumigatus* *A. fumigatus* strain 3.0772 was purchased from the China General Microbiological Culture Collection Center (Beijing, China). The culture medium was sabouraud，and the time was 3-4d. When the conidia are all over the surface, scrape it off and put it into PBS solution. Adjust its concentration to 5×10^4 conidia/mL.

Animal Preparation Adult (8-week) female C57BL/6 mice [specific pathogen-free (SPF)] were purchased from Jinan Pengyue Experimental Animal Co., Ltd. (Jinan, Shandong Province, China). The 8% chloral hydrate was used as an anesthetic, then one eye of these mice was infected by injecting spore suspension of *A. fumigatus* (0.5×10^6/mL) into the corneal stroma. Separated all the mice into two groups. One was treated with ASX (64 μmol/L), another group was treated with 1% dimethyl sulfoxide (DMSO) within PBS as control. Mice in different groups were given eye drops three times a day at the same time. Besides, the mice were not go through other treatment. Mice were examined every 24h post infection (p.i.). Scoring system of keratitis was determine in Wu *et al*[18]. RT-PCR, Western blot, MPO assay, and immunofluorescence staining were performed on cornea at the planned time.

RNA Isolation and RT-PCR The mRNA levels of various factors in mice cornea were detected by RT-PCR. Put the sample into RNAiso plus reagent to get total RNA. After quantification, RNA was reverse transcribed into cDNA, and then amplified by PCR using cDNA as template to obtain the mRNA expression of each factor. β-actin was used as control. The primer pair sequences were as follows: mβ-actin (F-GAT TAC TGC TCT GGC TCC TAG C and R-GAC TCA TCG TAC TCC TGC TTG C), mIL-1β (F-CGC AGC AGC ACA TCA ACA AGA GC and R-TGT CCT CAT CCT GGA AGG TCC ACG), mTNF-α (F-ACC CTC ACA CTC AGA TCA TCTT and R-GGT TGT CTT TGA GAT CCA TGC), mTLR-4 (F-CGC TTT CAC CTC TGC CTT CAC TAC AG and R-ACA CTA CCA CAA TAA CCT TCC GGC TC), mHMGB-1 (F-TGG CAA AGG CTG ACA AGG CTC and R-GGA TGC TCG CCT TTG ATT TTG G), mTLR-2 (F-CTC CTG AAG CTG TTG CGT TAC and R-TAC TTT ACC CAG CTC GCT CAC TAC).

Western Blotting The samples were first ground and centrifuged to remove the residue. Protein concentration of the samples were measured. These protein were transferred to PVDF membranes, followed by the incubation with primary antibodies against β-actin, TLR-4 (Abcam, USA) and TLR-2 (Abcam, Cambridge, UK) at 4℃ for 12-16h. Then the second antibody was added (37℃ for 1.5h), and the imaging was carried out after bath with ECL kit.

ELISA The expression of TNF-α, IL-1β and HMGB-1 was determined by ELISA. Corneas were removed at day 3 and dissoluted in 495 μL PBS solution with 5 μL protease inhibitor cocktail, then supernatant was carried out after ultrasonic dispersion. ELISA kits were used to quantify the protein concentrations from different groups. The results were carry out by absorbance.

Immunofluorescence Staining The eyeballs of mice were removed at appropriate time and fixed with liquid nitrogen. The sections were treated with serum, covered with primary antibody and incubated at room temperature for 30min, washed and incubated with secondary antibody, labeled with FITC, and stained with DAPI. Imaging was performed after closure. Fluorescence was examined under a Zeiss HB050 inverted microscope system (Carl Zeiss, Oberkochen, Germany).

Myeloperoxidase Assay Take 0.9 mL of 5% tissue homogenate, add 0.1 mL of reagent III, mix and bath at 37℃ for 15min. The determination group and the control group were prepared, fully mixed, 37℃ water bath for 30min, adding coloring reagent, 60℃ water bath for 10min, after taking out, immediately measure the OD value of each sample at 460 nm with the optical diameter (1 cm). The activity of neutrophils was calculated by the prescribed formula.

Statistical Analysis All numerical data were presented as means±standard errors of the mean (SEM). All experiments were performed at least three times. One-way analysis of variance (ANOVA) test was used to make comparisons among three or more groups, and unpaired two-tailed *t*-test also was used to identify the difference between multiple groups. *P* value less than 0.05 was defined to be statistically significant.

RESULTS

Clinical Response with ASX Treatment in *A. fumigatus* Keratitis Mouse Model In mice model with *A. fumigatus* keratitis, the corneas were more transparent in ASX-treated

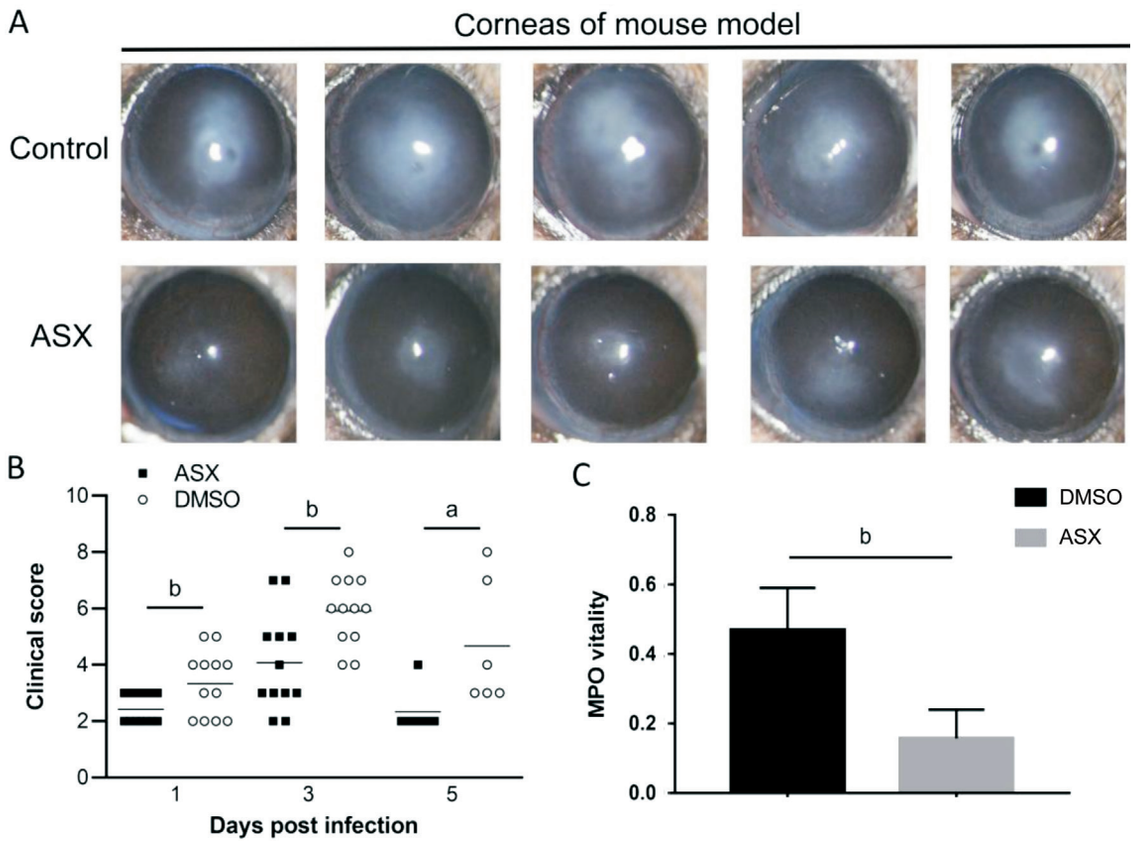

Figure 1 Clinical response and MPO with ASX treatment in a mouse *A. fumigatus* keratitis model A: Images captured with a slit lamp at day 3 p.i. (72h since the fungal spore suspension were inoculated on the cornea, the time when clinical response was most significant) illustrate the disease response of DMSO versus ASX-treated mice; B: Disease response is represented by a clinical score (n=12/group or n=6/group) which was higher for DMSO group compared with ASX-treated mice; C: Corneas (n=5/group) from ASX-treated mice exhibited a significant decrease in MPO level at day 3 p.i. compared with DMSO-treated mice. $^{a}P<0.05$, $^{b}P<0.01$.

group than control at day 3 (72h after model establishment; Figure 1A). The reason for choosing day 3 is that the disease condition of *A. fumigatus* keratitis model is the most significant on the day 3, and then it will be alleviated slowly. Generally, it can self heal on the fifth day. Clinical score in ASX treated group was lower than control group at days 1, 3, 5 (Figure 1B). These results indicated that ASX could improve corneal transparency and may exert protective effects in FK. In addition, MPO vitality was significantly reduced in corneas of ASX-treated mice compared with control (P<0.01) at day 1 (Figure 1C), suggesting ASX played an anti-inflammatory role in *A. fumigatus* keratitis.

ASX Treatment Reduced Recruitment of Neutrophils in the *A. fumigatus* Keratitis Mouse Mode We then detected the effects of ASX on the neutrophil infiltration in *A. fumigatus* keratitis mice. Immunofluorescence revealed the number of neutrophils were significantly decreased in ASX-treated mice compared with DMOS-treated group at day 3 (Figure 2), suggesting ASX suppressed the neutrophil infiltration in inflammatory response.

ASX Inhibited Production of *A. fumigatus*-induced Inflammatory Cytokines In the cornea of *A. fumigatus* keratitis mice, mRNA expressions of TNF-α, IL-1β, and HMGB-1 mRNA were detected at days 1, 3, and 5 after ASX treatment. The expression levels of IL-1β, TNF-α were significantly lower in ASX-treated groups than DMSO-treated group at day 1, 3, and 5 (Figure 3A, 3B). While HMGB1 only showed statistically difference at day 3 (Figure 3C). Additionally, ELISA showed the decreased expression level of TNF-α, IL-1β, and HMGB-1 in ASX group at day 3 (Figure 3D-3F).

ASX Inhibited the Elevated Expression of TLR4 and TLR2 To investigate whether TLRs are involved in ASX mediated anti-inflammatory activity, we tested the expression of TLR2 and TLR4 in *A. fumigatus* infected keratitis mouse model. The relative mRNA levels of TLR2 at days 3 and 5 were significantly down-regulated in ASX-treated group compared with DMSO group (Figure 4A-4C), and the relative mRNA levels of TLR4 at days 1, 3, and 5 were also reduced in ASX-treated group (Figure 4D-4F). In addition, Western blot showed that the expressions levels of TLR2 and TLR4 were enhanced upon *A. fumigatus* infection, which were significantly suppressed by ASX treatment at days 3 and 5 (Figure 4G, 4H). These results confirmed the inhibitory function of ASX

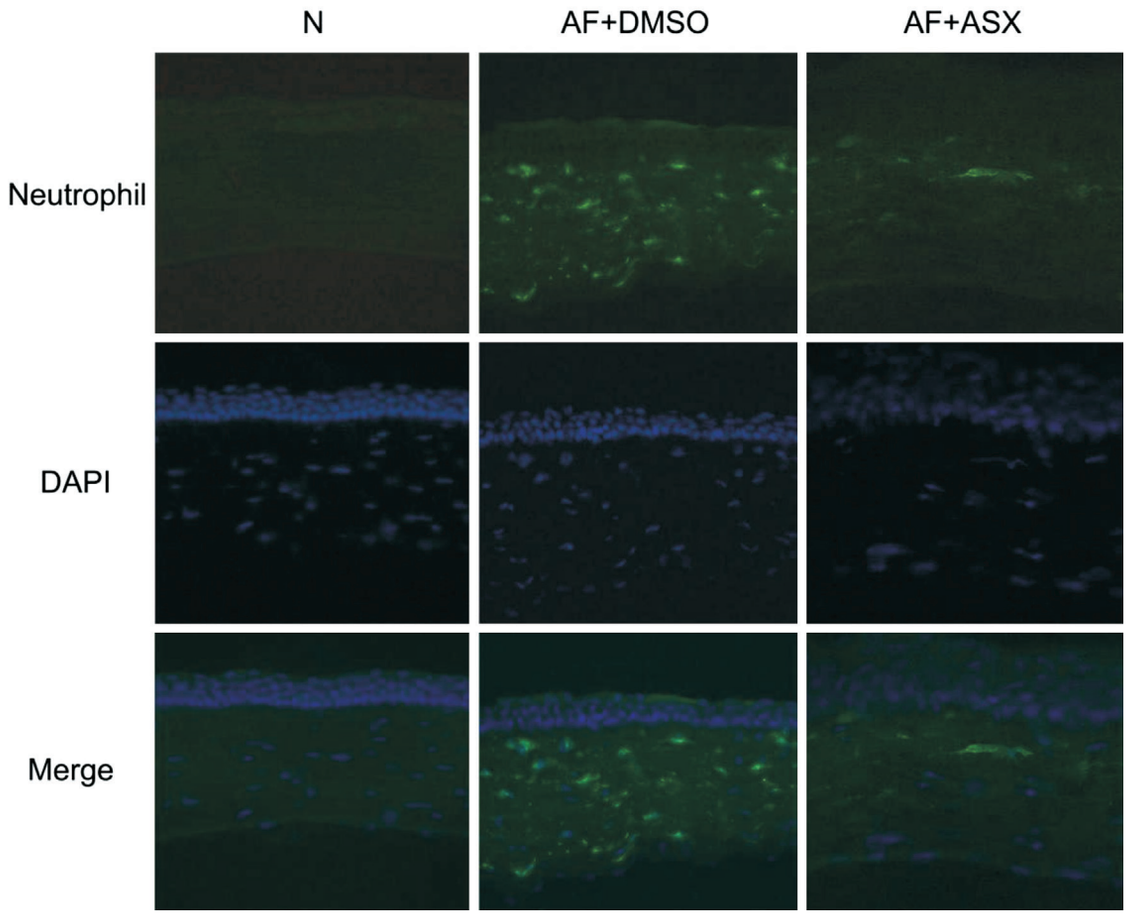

Figure 2 Results of immunofluorescence in the mice *A. fumigatus* keratitis model Positive staining (green) of the corneas of ASX-treated mice demonstrate decreased of neutrophil infiltration compared with DMSO-group at 3d p.i.

on TLRs, which may affect the neutrophil and macrophage infiltration.

DISCUSSION

FK is a tremendous challenge in ophthalmologic field, especially *A. fumigatus*[19-20]. Clinical outcome for FK is poor even with an appropriate timely treatment. One of the most important reasons is the excessive innate immune response that recruits various of inflammatory cytokines and immune cells, which promotes protein precipitation, reducing corneal transparency[21-23]. Therefore, it is crucial to control the inflammatory response in the middle and late stage of FK where the tissue damage caused by excessive inflammatory response is greater than its protective effect.

We investigated the anti-inflammatory function of ASX in FK. ASX is a strong anti-oxidant carotenoid that plays a lot of biological roles in hepatitis, pancreatitis, mastitis and uveitis[10,13-14,24-27]. We showed that ASX significantly improved prognosis by increasing corneal transparency, suggesting ASX may have a protective role in FK. In other words, ASX may prevent protein precipitation in cornea by reducing immune cells recruitment, or suppressing the release of inflammatory cytokines.

Neutrophils have cause of chemotaxis and phagocytosis, which promote neutrophils and macrophages to quickly infiltrate into infected tissues and phagocytose the microbes[28-29]. However, the excessive aggregation of neutrophils and macrophages could damage the integrity of corneal stroma and endothelium, which is the key to maintain the corneal transparency[30]. In our study, ASX treatment significantly decreased MPO level and depressed neutrophil infiltration, which therefore reduce the protein precipitation to protect the transparency of cornea. Dolma *et al*[13] revealed a therapeutic effect of ASX on infectious mastitis, in which ASX-treated mice had a normal clinical score but less neutrophil infiltration in breast tissue section. Therefore, we believe that ASX can effectively reduce the aggregation of neutrophils during the process of *A. fumigatus* keratitis, thus protecting corneal tissue and achieving a therapeutic effect on the disease.

Inflammatory cytokines like TNF-α and IL-1β are important indicators that reflect the severity of inflammatory response. Previous studies[31-33] suggested that ASX suppressed the expression of inflammatory factors in acute inflammation. Suzuki *et al*[14] found that ASX had inhibition effect for IL-1β, IL-6, and TNF-α through blocking the NF-κB dependent signaling pathway in rat uveitis. Zhou *et al*[34] found that ASX prevented tissue damage by down-regulating IL-1β and TNF-α

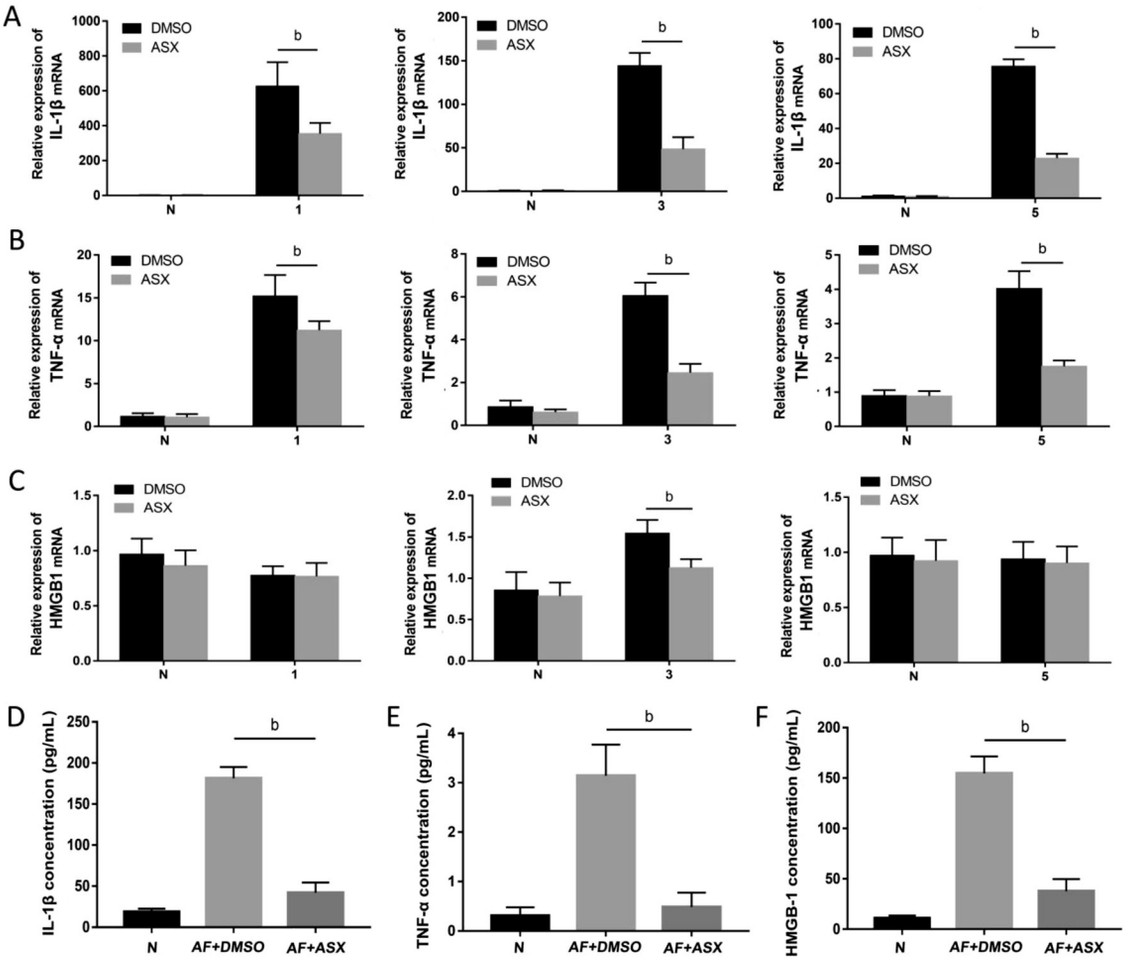

Figure 3 ASX inhibited production of inflammatory cytokines in the mice *A. fumigatus* keratitis model A-C: There was a significantly down-regulated for the relative expression of IL-1β and TNF-α after ASX-treatment in the mice model at days 1, 3, 5 p.i. For HMGB-1, the down-regulation was shown at day 3, but not shown at days 1, 5 p.i. $^{b}P<0.01$. D-F: As it shown in the result of ELISA, the protein level of IL-1β, TNF-α, and HMGB-1 was remarkable down-regulated in ASX treatment group compare with DMSO-treated group at day 3. $^{b}P<0.01$.

in the sepsis model in mice. These conclusions are consistent with our results shown that ASX reduced expressions of IL-1β and TNF-α *in vivo*, which may reduce the severity of FK. HMGB1 can induces local inflammation by stimulating the overexpression of inflammatory cytokines, on the other hand, it can cause the inflammatory cascade reaction, result in aggravating the severity and increasing the duration of inflammation[35]. As an important inflammatory factor in FK, HMGB1 were found that it gradually increased in the keratitis animal model, and reached the peak at 3d p.i. In our study we demonstrate that expression levels of HMGB1 were lower in ASX-treated groups than controls, indicating ASX can effectively reduce the inflammatory response and protect the corneal tissue.

The innate immune system is closely related to inflammation in the pathogenesis of FK, it recognizes and eliminates fungi through PRRs[36-37]. PRRs recognize PAMPs in pathogen and mediate the neutrophil recruitment, chemokines production and phagocytosis, participating in the formation of the first defensive line against fungal infection[38]. PRRs also recognize the damage associated molecular patterns (DAMPs), which are released by stressed cells as alarmins, promoting inflammatory response[39]. TLR is one of the most important PRRs in the early recognition of PAMPs and DAMPs[40-41]. TLR2 and TLR4 were exist on cell surface, and had the function of identifying many factors[15]. In FK, TLR4, and TLR2 have been shown crucial to trigger chemokines such as IL-6, IL-1β, and TNF-α[42-43]. After TLRs recognized fungi, many inflammatory factors increased through different signaling pathways[16-17]. And the degree of the increase of inflammatory cytokins is in direct proportion to the severity of disease[44]. However, an excessive innate immune response release a large number of chemokines and cytokines to recruit various immune cells, resulting in protein precipitation and the loss of corneal transparency[45]. Thus, control innate immune response is important to maintain corneal transparency[46]. Many studies have shown that ASX could inhibit acute inflammation through TLRs in various tissues such as hepatitis, mastitis and nervous system[47-49]. But for the eye infections, the effect of ASX is still unclear. Our study demonstrated that ASX suppressed the

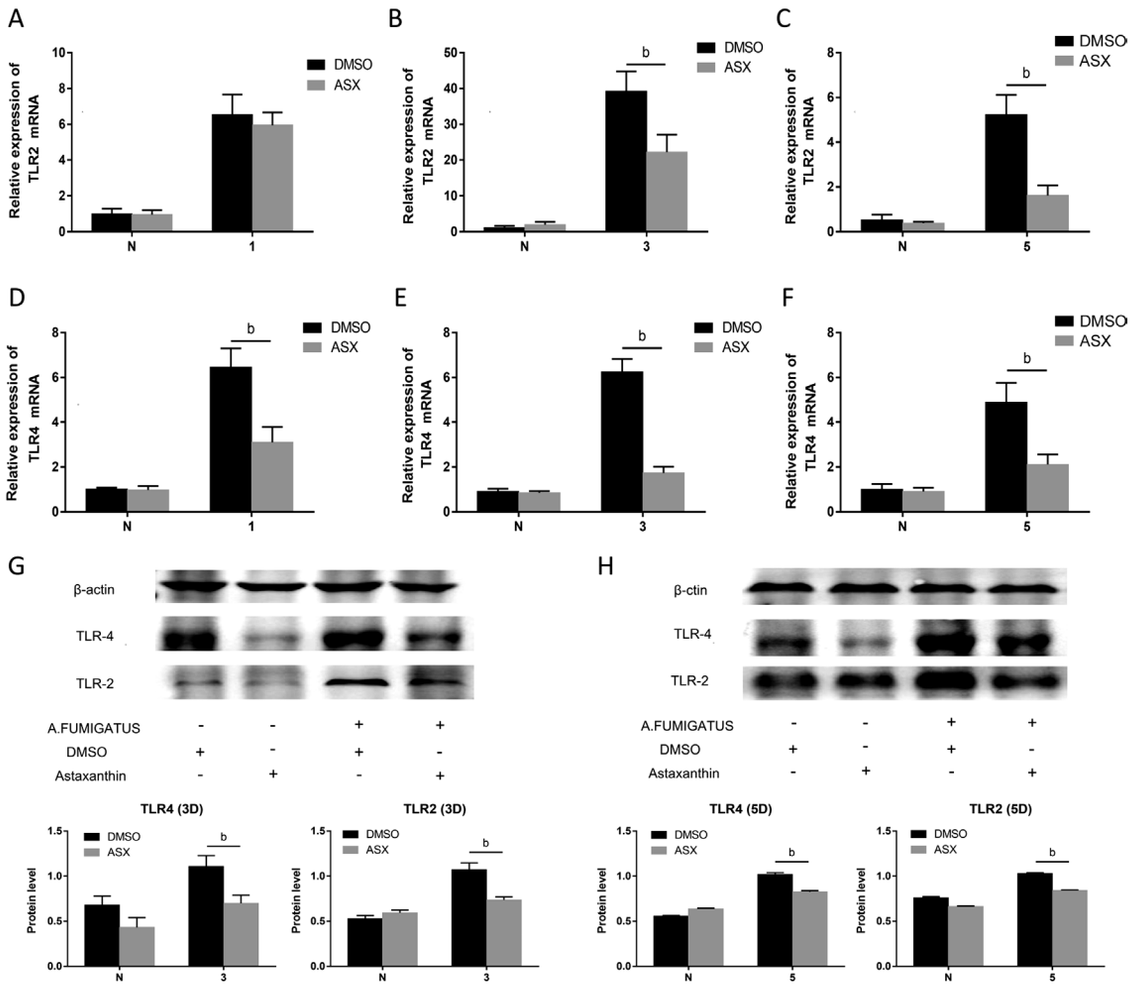

Figure 4 ASX inhibited the TLR4 and TLR2 expression which elevated in the mice model A-C: After treated with ASX, the relative expression of TLR2 was significantly down-regulated in the mice model at 3/5d p.i. (B, C), but the difference was not shown at day 1 (A; *n*=4/group). D-F: There was a significantly down-regulated for the relative expression of TLR4 after ASX-treatment in the mice model at days 1, 3, 5 p.i. (*n*=4/group). G, H: Compared with DMSO-treated group, TLR2 and TLR4 protein levels in corneas were significantly lower at days 3, 5 p.i. in ASX-treatment group. $^{b}P<0.01$.

A. fumigatus induced expressions of TLR2 and TLR4 *in vivo*, and significantly attenuated corneal inflammation. TLR2 and TLR4 were down-regulated in ASX-treated group compared with DMSO group in mice model. TLR2 and TLR4 increased gradually after fungal infection in cornea, and reached a high level at 3d p.i., which also coincided with the clinical observation that the most serious corneal inflammatory reaction was observed on the third day in *A. fumigatus* infection. After 3d p.i., the clinical manifestations of the disease gradually alleviate, and the levels of TLR2 and TLR4 were decreased, which is shown by the results in the fifth day. In this process, ASX significantly inhibited the expression of TLR2 and TLR4. Thus, ASX could be a potential drug that targets and inhibits TLRs function to prevent tissue damage caused by excessive inflammatory response in FK. Further studies may focus on the underlying mechanism of the inhibitory effects of ASX on TLRs, and how it regulates the downstream pathways.

Therefore, ASX may be a potential, targeted drug to inhibit TLRs in order to prevent tissue damage caused by excessive inflammatory response of FK. Although our data show that ASX plays a major role in TLRs in corneal tissue, its protective effect on corneal tissue in FK may not only depend on the inhibition of TLRs activation. The complexity of this mechanism is a common problem in clinical and experimental pharmacology research, because drugs rarely act on only one molecular target. So the comprehensive and detailed mechanism of drug function is worth studying. However, it can be concluded that ASX can effectively inhibit the production of inflammatory factors in FK, protect corneal tissue, increase corneal transparency.

In summary, ASX can significantly improve the clinical manifestations of *A. fumigatus* keratitis in mice by reducing immune cells recruitment and suppressing the release of inflammatory cytokines. Thus, ASX could be a potential drug used to inhibit inflammation in FK, protecting corneal

transparency and preventing visual loss. In order to evaluate more about its clinical value, future study should explore the effect of ASX in combination with other anti-fungal drugs, and compare the effect of ASX with common anti-inflammatory drugs which commonly used to treat FK.

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Conflicts of Interest: Huan Y, None; **Peng XD,** None; **Lin J,** None; **Zhang YX,** None; **Zhan L,** None; **Gao H,** None; **Zhao GQ,** None.

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