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Nerolidol inhibits the LOX-1 / IL-1β signaling to protect against the *Aspergillus fumigatus* keratitis inflammation damage to the cornea☆

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ABSTRACT

Purpose: Nerolidol, a naturally occurring sesquiterpene has both anti-microbial and anti-inflammatory properties. The current study aims to investigate the antifungal and the anti-inflammatory effects of nerolidol against mouse *Aspergillus fumigatus* (*A. fumigatus*) keratitis.

Methods: The minimum inhibitory concentration (MIC) and cytotoxicity tests were used to study the antifungal ability. For in vivo and in vitro studies, the mouse corneas and the human corneal epithelial cells (HCECs) infected with *A. fumigatus* spores were intervented with nerolidol or phosphate buffer saline (PBS). Thereafter, the effect of the nerolidol on the response against inflammation was analyzed using the following parameters: recruitment of the neutrophils or macrophages and the expression of the lectin-type oxidized low density lipoprotein receptor-1 (LOX-1) and interleukin 1β (IL-1β). Techniques used were the slit lamp, immunofluorescence, myeloperoxidase (MPO) detection, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot.

Results: Nerolidol directly inhibits the growth of *A. fumigatus*. The administration of nerolidol reduced the severity of fungal keratitis with infiltration of fewer inflammatory cells and reduced levels of the LOX-1, as well the anti-inflammatory cytokines such as IL-1β were reduced compared with the PBS group. Additionally, in vitro studies showed that treatment with nerolidol inhibited the production of the LOX-1 / IL-1β levels in *A. fumigatus* stimulated HCECs.

Conclusion: Nerolidol attenuated the *A. fumigatus* keratitis inflammatory response by inhibiting the growth of *A. fumigatus*, reducing the recruitment of the neutrophils and the macrophages, and inhibiting the LOX-1/ IL-1β signaling.

1. Introduction

Fungal keratitis is an infectious eye disease caused by the pathogenic fungi like *A. fumigatus* and *Fusarium*. It results in difficult to treat corneal ulcers, which often lead to blindness [1,2]. The most common pathogenic bacteria in China is the plant-associated fungi *A. fumigatus* [3].

After corneal infection, the pattern recognition receptors (PRRs) of the cornea interacts with the fungal pathogen-associated molecular pattern (PAMP), initiating the innate immune response against the fungal pathogens. This leads to the recruitment and aggregation of a large number of neutrophils and chemokines causing edema, inflammatory infiltration, ulceration and perforation of the cornea. Thus, an excessively strong immune response against the foreign infection causes serious damage, making it difficult for the corneal tissues to recover back its transparency [4,5]. Therefore, researchers are interested to explore anti-fungal and anti-inflammatory drugs which can protect the cornea against fungal infections while avoiding excessive inflammatory reactions.

Nerolidol, a natural sesquiterpene found in a variety of flower and plant essential oils [6] has recently been used widely in many industries due to its easy access and insignificant side effects [7]. Studies have

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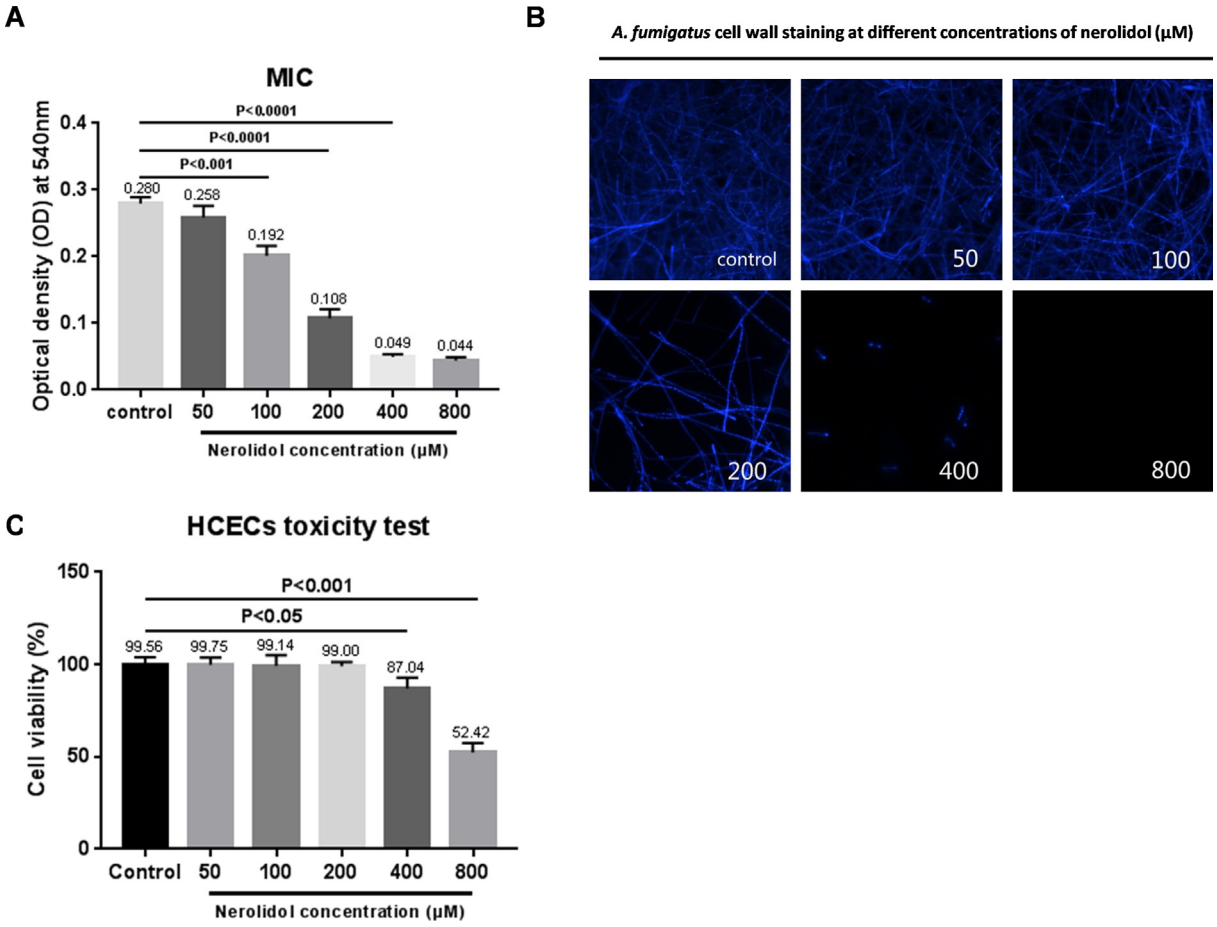

Fig. 1. Nerolidol has an inhibitory effect on *A. fumigatus* and cause damage to cells at certain concentrations. The OD value (n = 6/group) was measured at 540 nm after incubating the conidia with different concentrations of nerolidol for 24 h (Fig. 1A). Results show nerolidol has an inhibitory effect on *A. fumigatus* at 100 μM, and the inhibitory effect is dose-dependent. Fluorescent staining of the cell wall of the hyphae (Fig. 1B), show that nerolidol completely inhibits the fungal growth at 800 μM. The cytotoxicity test of the HCECs (Fig. 1C) (n = 6/group) showed that nerolidol damages the cell viability of the HCECs at 400 μM compared to the control group ($P < 0.05$).

shown its potent biological activities, viz., antibacterial, anti-inflammatory, antioxidant, anti-tumor etc [8–13]. However, the role of nerolidol in the prevention and treatment of fungal keratitis has not yet been studied. Thus, in the current study, we aim to investigate the potential role of nerolidol as a possible therapeutic drug against *A. fumigatus* keratitis.

2. Materials and Methods

2.1. Minimum inhibitory concentration (MIC) of nerolidol on A. Fumigatus

The *A. fumigatus* strain 3.0772 was supplied by the China General Microbiological Culture Collection Center (Beijing, China). After 2–3 days of culture, the conidia were scraped into PBS, and the volume was adjusted to 5×10^6 cfu/ml for subsequent use.

For the MIC test, the conidia suspension was adjusted to 5×10^3 cfu/ml with the Sabour culture media. Nerolidol (Selleck Chemicals) was dissolved in the adjusted conidia/Sabour culture for a two-fold dilution, the maximum concentration was 800 μM and the minimum concentration was 50 μM. Different concentrations of the nerolidol mixture were dispensed into a 96-well plate to a volume of 100 μl per well. The 96-well plate was cultured in a 37 °C incubator for 24 h, and the optical density (OD) was measured with a microplate reader at 460 nm. Then, the 96-well plate was centrifuged, the supernatant discarded, and the hyphae cell wall was fluorescently stained by adding Calcofluor White Stain (Sigma-aldrich) to each well. The plate was incubated for another 30 min following which digital images were captured with the Zeiss Axiovert microscope at × 100 magnification.

2.2. Toxicity test of nerolidol on the human corneal epithelial cells (HCECs)

Immortalized HCECs were bought from the Ocular Surface Laboratory (Zhongshan Ophthalmic Center). 100 μl of the HCECs suspension was placed in a 96-well plate. The plates were incubated in an incubator (37 °C, 5% CO_2) till 80% confluency. After this, the original liquid in the well was removed, and 100 μl of different concentrations of the nerolidol/F12 medium were added to each well. In the control group, 100 μl of F12 medium without nerolidol was added. The plates were incubated for another 24 h in the incubator. 10 μl of the Cell Counting Kit-8 (Solarbio, Beijing, China) was added to each well, and the plate was incubated again for 2 h. After this, the 96 well plate was read with a microplate reader at 450 nm.

2.3. Mice model of A. fumigatus keratitis

Eight-week-old C57BL/6 female mice (Jinan Pengyue Laboratory Animal Ltd., Jinan, China) were used for the study. After being anesthetized with 8% chloral hydrate (0.08 ml/mouse) solution intraperitoneally, the mice were injected with a 2.5 μl solution of the *A. fumigatus* conidia suspension (2.5×10^6 cfu/ml) into the right corneal stroma with a micro-injector. The mice treatments were in compliance with the regulations of the Chinese Ministry of Science and Technology

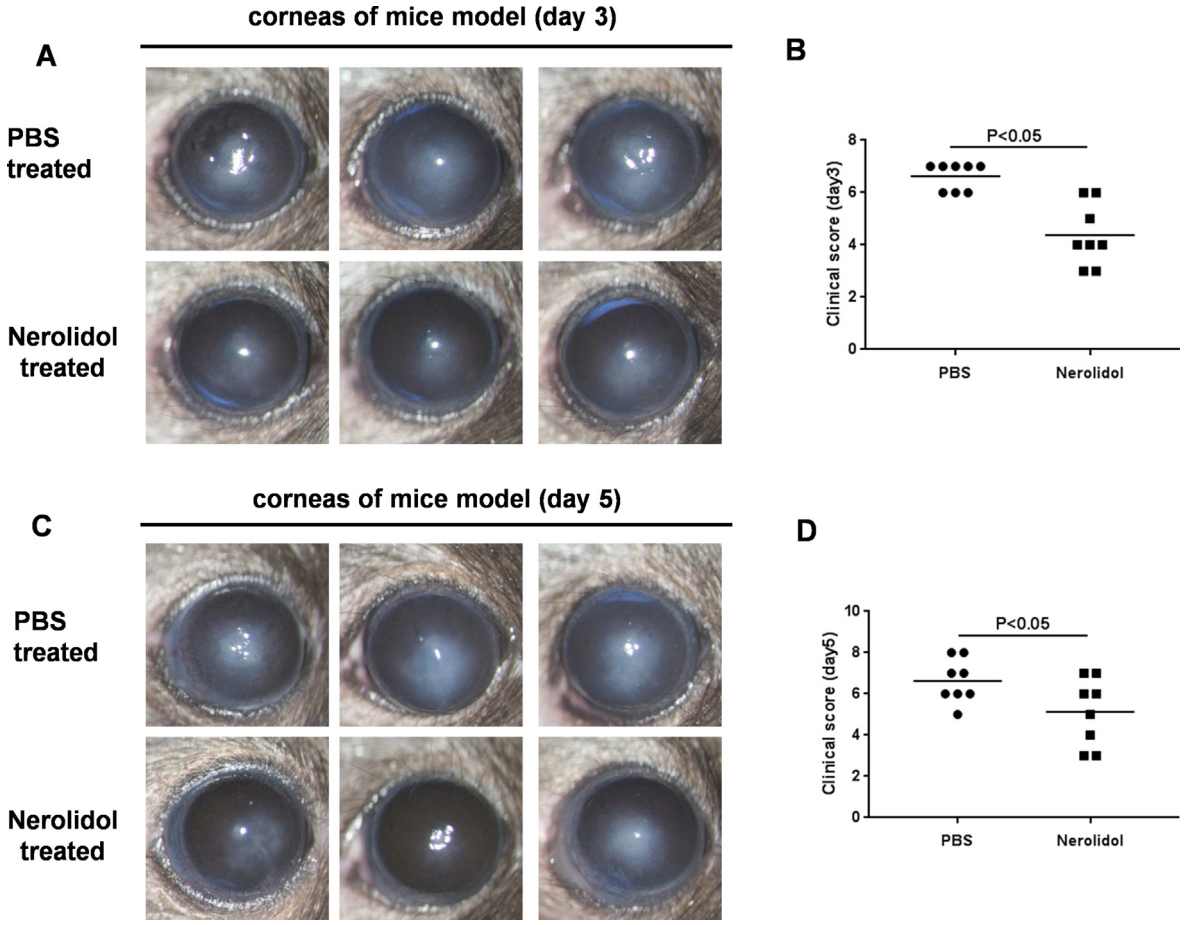

Fig. 2. Nerolidol reduced the inflammatory response in the *A. fumigatus* infected corneas (n = 8/group). The images show that the nerolidol treatment group had a higher corneal transparency, less edema, and less inflammatory infiltration after 3 days (A) and 5 days (C). Clinical scores in the nerolidol treated group were lower than those of the PBS control group after 3 days (B) and 5 days (D), and the difference was statistically significant ($P < 0.05$).

Guidelines on the Humane Treatment of Laboratory Animals (vGKFCZ-2006–398) and with the Statement on the Use of Animals in Ophthalmic and Vision Research declared by the Association for Research in Vision and Ophthalmology (ARVO).

2.4. Rescue of the mouse cornea and the HCECs

The mice underwent various time dependent rescue attempts (after 2 h, day – 1 or 3 or 5 days) after the right cornea infection by *A. fumigatus* by a subconjunctival injection of 5 μl of nerolidol dissolved in PBS (200 μM) or with PBS alone. The mouse corneas was observed and photographed with a slit lamp every day. Ocular disease was graded using clinical scores according to the scoring system proposed by Wu et al. [14]. The eyeball or corneas of the mice sacrificed on the 2nd day were checked for immunofluorescence and MPO, while the cornea of the mice sacrificed on the 3rd day were analyzed with the qRT-PCR and Western blot. The *A. fumigatus* conidia (5×10^6 cfu/ml) stimulated HCECs grown to a density of 80% confluency were treated with either nerolidol (200 μM, 5 μl) or PBS (5 μl) and incubated for 8 or 16 h in an incubator. They were then used for qRT-PCR or Western blot studies, respectively.

2.5. Immunofluorescence staining

Eyeballs were embedded in the optimal cutting temperature (O.C.T.) (Sakura Finetek USA, Inc.) media and rapidly frozen with liquid nitrogen. They were then cut with a cryostat at a thickness of 10 μm. The tissue slices were blocked with 10% animal serum (Solarbio, Beijing, China) for 30 min at room temperature. To label the neutrophils and macrophages, the slices were incubated with either the NIMP-R14 antibody (Santa Cruz Biotechnology) or the F4/80 antibody (Cell Signaling Technology) at 4 °C overnight. After being washed with PBS, the tissue sections were stained with the CY3 conjugated IgG (Elabscience, Wuhan, China) for 1 h and then DAPI solution (Solarbio, Beijing, China) for another 10 min. Finally, the slices were observed under the Zeiss Axiovert microscope and digital images were captured at × 200 magnification.

2.6. Quantitation of the mice corneal neutrophils

Myeloperoxidase (MPO) is unique to neutrophils, and its activity is used as a method for quantifying neutrophil levels. The corneal tissue placed in the working fluid of the MPO kit (Njjcbio, Nanjing, China) were incubated in a water bath at for minutes. Following the incubation, the MPO activity of the tissues was read at 450 nm OD using a microplate reader.

2.7. Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA of corneas and HCECs were extracted using the RNAiso Plus kit (Takara, Dalian, China), and cDNA was obtained by reverse transcription of total RNA using the Primescript RT kit (Takara, Dalian, China). Finally, qRT-PCR was performed was performed by Eppendorf Mastercycler and SYBR green. β-actin and GADPH were used as internal reference. The sequences of the oligonucleotide primers are as follows: mLOX-1F-AGG TCC TTG TCC ACA AGA CTG G and R-ACG CCC CTG

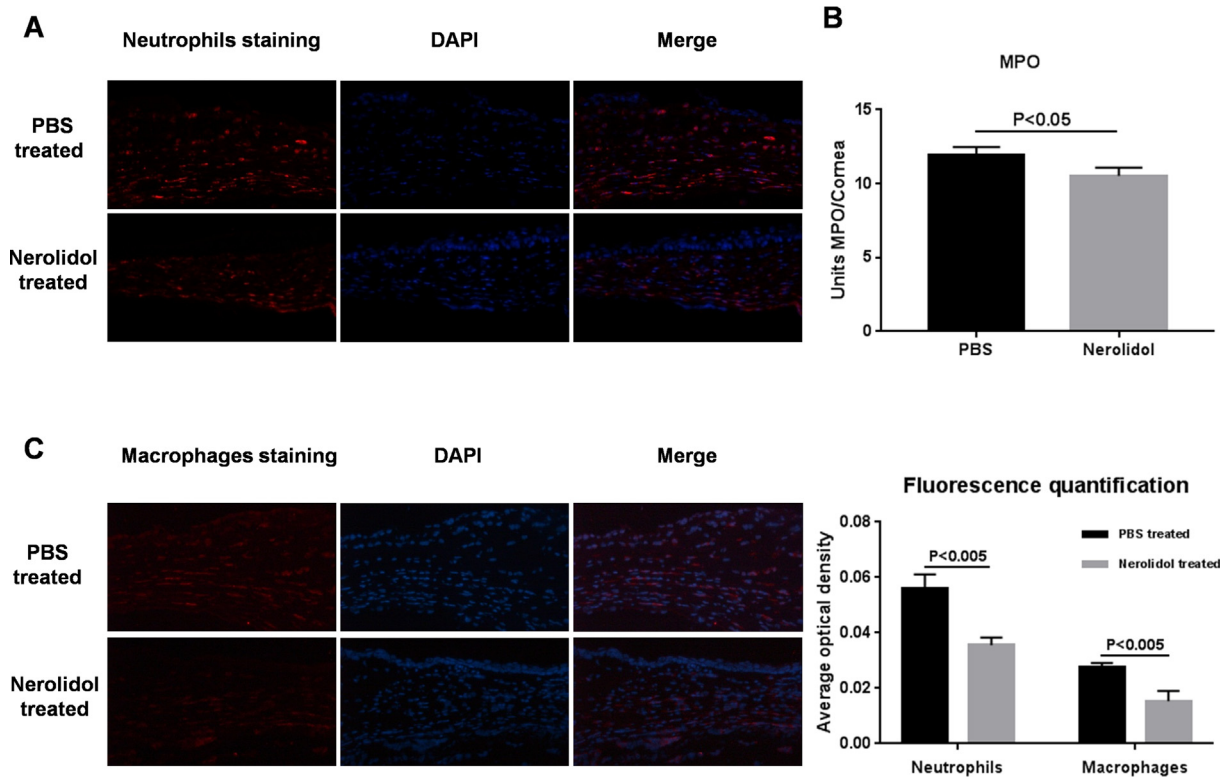

Fig. 3. Treatment with nerolidol reduced the inflammatory cells migrating to the corneal stroma after the *A. fumigatus* infection (n = 6/group). Neutrophils marked as red fluorescence with the NIMP-R14 (A), were quantified by detecting the activity of the MPO in the corneal tissue (B). Macrophages are marked as red fluorescence with the F4/80 (C), while the blue fluorescence represents DAPI. Compared with the PBS group, the numbers of the neutrophils and the macrophages decreased after the nerolidol treatment ($P < 0.005$).

GTC TTA AAG AAT TG, hLOX-1F-ATG ACC TAA AGA TCC AGA CTG TGA A and R-TGC ATG CCC AGC ACC ATA, mIL-1β F-CGC AGC AGC ACA TCA ACA AGA GC and R-TGT CCT CAT CCT GGA AGG TCC ACG, hIL-1β F-GCT GAT GGC CCT AAA CAG ATG AA and R-TCC ATG GCC ACA ACA ACT GAC, mβ-actin F-GAT TAC TGC TCT GGC TCC TAG C and R-GAC TCA TCG TAC TCC TGC TTG C, hGADPH F-GCA CCG TCA AGG CTG AGA AC and R-TGG TGA AGA CGC CAG TGG A.

2.8. Western blot

Total protein of corneas and HCECs was extracted using the tissue protein lysate (radioimmunoprecipitation assay: phenylmethanesulfonyl: phosphatase inhibitor = 100:1:1) (Solarbio, Beijing, China). Protein samples were separated by SDS-PAGE gels (12%) and transferred to PVDF membrane (Millipore). Put the membrane in blocking buffer (Beyotime, China) for 2 h. Incubated with primary antibody of target protein at 4 °C overnight, and secondary antibody for 2 h. Primary antibodies we were used: OLR1 Antibody (Proteintech), IL-1 beta Antibody (R&D), β-Actin mAb (Cell Signaling Technology). Secondary antibodies we were used: HRP-linked anti-rabbit (Elbscience) and anti-goat (Elbscience) antibodies. The bands were visualized with Western ECL Blotting Substrates (Bio-Rad). Digital images were obtained by a Vilber Solo 4S chemiluminescence imaging system.

2.9. Statistical analysis

Experimental data is expressed in the form of mean ± standard deviation. The analysis softwares uses GraphPad Prism (USA, GraphPad Software) and ImageJ2x (Inc. Germany, Rawak Software). MIC data, clinical scores, fluorescence quantification, MPO, qRT-PCR and Western blot were all unpaired two-tailed Student's *t* test. Differences in $P < 0.05$ were considered statistically significant.

3. Results

3.1. Nerolidol directly inhibits the growth of A. fumigatus and its toxicity towards the HCECs

The antifungal ability of nerolidol was demonstrated by the MIC test (Fig. 1A) and the hyphae cell wall staining (Fig. 1B). The 460 nm OD values showed that nerolidol significantly inhibited the growth of *A. fumigatus* at 100 μM compared to the control group ($P < 0.01$). The hyphal cell wall staining showed that the 800 μM concentration of nerolidol completely inhibited the germination of *A. fumigatus* spores. HCECs were used to detect the toxic effects of nerolidol (Fig. 1C). Cell viability results showed that nerolidol had a significant killing effect on the HCECs at 400 μM ($P < 0.05$) compared to the control group. Known from this, 200 μM is a safe and effective dose and used for all experiments in this study.

3.2. Disease response with the nerolidol treatment in mice A. fumigatus keratitis

The effect of nerolidol in reducing the inflammatory response of the mouse corneas was indicated by the slit lamp photographs (Fig. 2A, C) and the clinical scores (Fig. 2B, D). The slit lamp pictures showed that the nerolidol (200 μM, 5 μl) treatment significantly reduced the inflammatory response of the cornea compared to the PBS (5 μl) only treated group after both 3 and 5 days. Clinical scores of the mouse corneas in the nerolidol treated group was significantly lower than that of the PBS control group ($P < 0.05$).

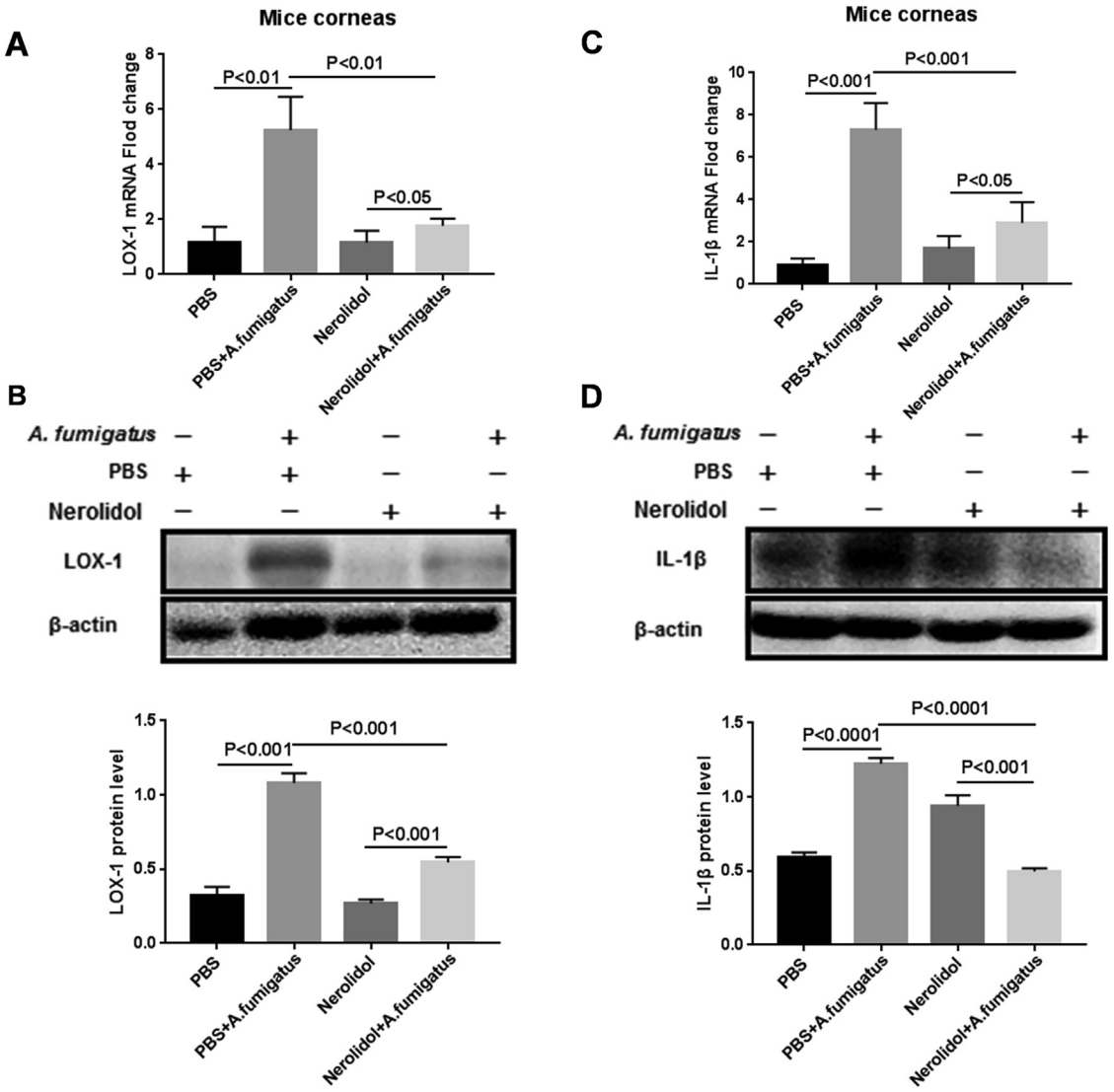

Fig. 4. Nerolidol inhibits the LOX-1 and IL-1β expression in *A. fumigatus* keratitis. After the mouse corneas (n = 6/group) were infected with *A. fumigatus* for 3 days, treatment of the corneas with nerolidol reduced the LOX-1 mRNA (A) and protein (B) levels in the cornea compared to the PBS treated group. Similarly, the LOX-1 and IL-1β mRNA (C) and protein levels (D) were significantly decreased after nerolidol treatment following 3 days of *A. fumigatus* infection.

3.3. Nerolidol reduced the infiltration of the neutrophils and the macrophages as also the MPO activity in mice A. fumigatus keratitis

Immunostaining and MPO assays were performed to detect the level of the neutrophils and the macrophages in mice *A. fumigatus* keratitis following nerolidol treatment. There was a marked reduction in the staining of NIMP-R14 (Fig. 3A) and F4/80 (Fig. 3C) in the nerolidol treated compared to the PBS control group, following 2 days of the mice corneal infection, indicating a decrease in the number of neutrophils and macrophages. The results showed that there was a significant decrease in the MPO activity in the nerolidol-treated group as compared to that of the control group ($P < 0.05$) (Fig. 3B).

3.4. Nerolidol inhibited the LOX-1/ IL-1β signaling in mice A. fumigatus keratitis

Following 3 days of nerolidol treatment the mRNA levels of both the LOX-1 (Fig. 4A, $P < 0.01$) and the inflammatory factor IL-1β (Fig. 4C, $P < 0.001$) as also the protein levels of both the LOX-1 (Fig. 4B, $P < 0.001$) and IL-1β (Fig. 4D, $P < 0.0001$) were reduced after 3 days of nerolidol treatment following post-infection.

3.5. Nerolidol inhibited the LOX-1/ IL-1β signaling in the HCECs stimulated by A. fumigatus

As in vivo studies, in vitro treatment of the *A. fumigatus* stimulated HCECs with nerolidol significantly decreased the mRNA levels of both the LOX-1 (Fig. 5A, $P < 0.0001$) and IL-1β (Fig. 5C, $P < 0.001$) compared to the PBS-treated group after 8 h. Additionally, following infection and 16 h of treatment of nerolidol, the protein levels of LOX-1 (Fig. 5B, $P < 0.01$) and IL-1β (Fig. 5D, $p < 0.001$) were also significantly reduced as compared to the PBS-treated group.

4. Discussion

Fungal keratitis is a type of infectious keratopathy that is both difficult to diagnose and treat [15]. Clinically, there are many patients with fungal keratitis who develop corneal perforation, eventually losing their eyeballs [16]. Therefore, it is necessary to find a drug to inhibit corneal inflammation. In the current study, we investigated the function of nerolidol in controlling fungal keratitis by using a mouse model of *A. fumigatus* keratitis.

A. fumigatus is one of the most common pathogens causing corneal infections. When the immune barrier of the cornea is destroyed, the

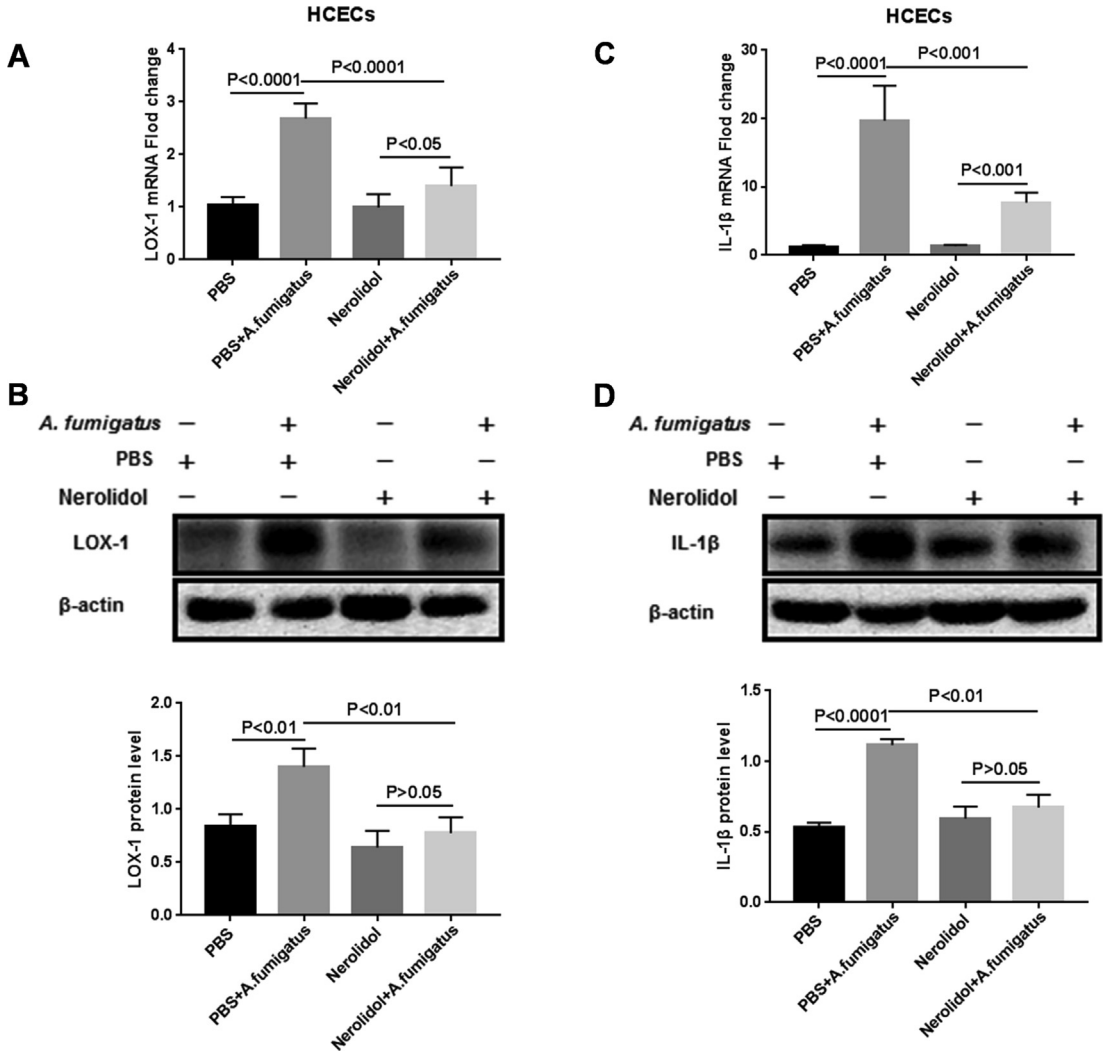

Fig. 5. Nerolidol inhibits the LOX-1 and IL-1β expression levels in the *A. fumigatus* stimulated HCECs (n = 6/group). Treatment with nerolidol reduced both the mRNA (A) and protein (B) levels of the pattern recognition receptor LOX-1 compared with the PBS group in *A. fumigatus* stimulated HCECs. Similarly, IL-1β mRNA (C) and protein (D) levels were decreased after nerolidol treatment to the HCECs stimulated by *A. fumigatus* as compared to the PBS group.

pathogenic fungus can attach to the cornea, thereby releasing toxic metabolites which infiltrates and destroys the corneal tissue [16]. In our experiments, we observed that nerolidol inhibits the proliferation of *A. fumigatus*. Dose-dependent nerolidol inhibition of *A. fumigatus* in HCECs showed that it significantly inhibited the growth of the *A. fumigatus* hyphae at 100 μM and a complete inhibition at 800 μM. Studies (Curvelo JA et al. [9]) have shown that nerolidol inhibits the growth, transition (yeast to hyphae), formation and stability of biofilms produced by C. albicans. Further, Pontin M et al. [17] observed that nerolidol significantly reduced the production of sclerotia to inhibit the fungal growth. The difference in the mycelial uptake of ethidium bromide (EtBr) after nerolidol treatment suggests that one of the antifungal mechanisms of nerolidol might be the destruction of the fungal membrane integrity. Based on these observations, we hypothesize that nerolidol protects the cornea by limiting the growth of *A. fumigatus*.

As the corneal epithelial cells act as the first line of defence against fungal infections, we used the HCECs to study the toxicity of nerolidol. The in vitro results showed that 200 μM is the max nerolidol concentration to have an antifungal effect which was not toxic to the HCECs. The in vivo study confirmed 200 μM to the effective nerolidol dose as this dose didn't damage the cornea. Thus, in the current study, 200 μM is used for all experiments as it is a safe and effective dose.

Previous studies has shown nerolidol to have protective properties against various inflammatory diseases such as acute kidney injury and acute peritonitis [10,11]. In the current study, topical application of nerolidol in the fungal keratitis mouse model significantly reduced the degree of edema and opacity of the cornea. Zhang L et al. [10] showed that the intraperitoneal injection of nerolidol inhibited the pathological damage induced by LPS in the rat kidneys, by reducing the edema, tubular dilatation and distortion of rat renal tubular epithelial cells in a dose-dependent manner. Further, Fonsêca DV et al. [11], study in the carrageenan-induced peritonitis model showed nerolidol reduced the influx of the polymorphonuclear cells to attenuate the level of the inflammatory factors in the peritoneal lavage. These results substantiate the anti-inflammatory protective properties of nerolidol in the mouse *A. fumigatus* keratitis model.

Another significant contribution of this study is the finding that nerolidol mediates its anti-inflammatory actions by inhibiting the recruitment of the neutrophils and the macrophages after *A. fumigatus* infects the cornea. Neutrophils and macrophages are important inflammatory cells that mediate the corneal antifungal immunity response. When the cornea is infected with fungi, neutrophils are attracted by the chemotactic substances released from the limbal vessels to the site of the infection [18,19]. The aggregated neutrophils infiltrating the corneal stroma account for more than 80% of all immune cell types [6], exerting chemotaxis, phagocytosis and bactericidal

actions. As the neutrophils initiated its phagocytosis action to engulf the fungal cells, the large amount of lysosomal enzymes contained within it, decomposed the fungi, thereby limiting and destroying the invading fungi from causing further damage to the cornea. However, following its phagocytosis phenomena, neutrophils secrete or release a large amount of toxic substances and oxygen free radicals, resulting in sustained inflammatory reactions which destroys the corneal structure, causing visual impairment and increased tissue damage [20–22]. Macrophages which are antigen presenting cells, phagocytose pathogenic microorganisms and secrete immune active substances to continue the immune reactions in inflammation [23,24]. After the corneal fungal infection, the macrophage action to phagocytose the pathogens while simultaneously releasing chemokines to recruit the granulocytes amplifies the corneal inflammatory response, causing more harm to the cornea from the edema, inflammatory infiltration, ulceration and perforation of the cornea [24–26]. These studies show that inflammatory cells are a “double-edged sword” [27,28]. Based on the above studies, we hypothesize that nerolidol reduces the corneal clinical score, possibly by inhibiting the recruitment of the neutrophils and the macrophages to the corneal stroma after *A. fumigatus* infection in the mice.

Additionally, we deciphered the anti-inflammatory molecular mechanism of nerolidol which acts by inhibiting the expression of the pattern recognition receptor LOX-1 and inflammatory factor IL-1β in mouse cornea at both the mRNA and protein levels. Since the corneal epithelial cells are the first barrier against the corneal infection, we stimulated the human corneal epithelial cells cultured in vitro with *A. fumigatus* to establish a cell model of fungal infection. The above results were verified in vitro and consistent with those in the animal model. LOX-1 belongs to the C-type lectin family and plays an important role in the initiation and development of inflammatory responses. In addition to its expression in the endothelial, smooth muscle, platelets and other cells, LOX-1 is also expressed in the macrophages and the neutrophils [29–32]. Previously it has been shown that LOX-1 is an important pattern recognition receptor involved in the corneal antifungal immune response [33,34]. Following inhibition of LOX-1, there is a reduction in the levels of intracellular ROS production, p38MAPK dephosphorylation, NF-κB translocation and Fas and FasL binding with corresponding increased BCl-2 expression, which in combination reduces inflammation [20,35]. IL-1β is an important inflammatory factor involved in the corneal antifungal immune response, produced mainly by the activated monocytes, which mediate the acute inflammatory response [36]. Studies have found that when *Pseudomonas aeruginosa* keratitis occurs in the IL-1β knockout mice, the infiltration of the inflammatory cells to the corneal infection area is significantly reduced [37]. Thus, combining all the above studies, we hypothesize that nerolidol exerts its anti-inflammatory action in the *A. fumigatus* keratitis by blocking the LOX-1/IL-1β inflammatory signal.

Thus, we can conclude that nerolidol reduces the inflammatory response of *A. fumigatus* keratitis by a 3 step process: (1) inhibits the fungal growth, (2) reduces the recruitment of the neutrophils and the macrophages and (3) inhibits LOX-1/IL-1β signaling. Therefore, nerolidol has potential therapeutic properties in controlling the severity of fungal keratitis. In the future, more studies will focus on its therapeutic effect combined with first-line antifungal drugs like natamycin and voriconazole.

5. Author contribution statement

Hua Yang, contributed to data acquisition, analysis, and interpretation, drafted and critically revised the manuscript;

Qian Wang, Lin Han, Xuejiao Yang, Wenyi Zhao, Leyu Lyu, Limei Wang, Haijing Yan, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript;

Chengye Che, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript.

All authors gave final approval and agree to be accountable for all aspects of the work.

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