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Wedelolactone suppresses IL-1β maturation and neutrophil infiltration in *Aspergillus fumigatus* keratitis☆

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ABSTRACT

Purpose: Wedelolactone, a chemical compound extracted from Wedelia calendulacea or *Eclipta alba*, has been reported to regulate key steps in inflammation. However, the effects of wedelolactone on fungal keratitis are not known. Hence, we aimed to characterize the impact of wedelolactone in *Aspergillus fumigatus* keratitis.
Methods: *Aspergillus fumigatus* was used to establish an in vivo mouse model of fungal keratitis and an in vitro model of THP-1 macrophages. Mice and THP-1 macrophages were pre-treated with wedelolactone. Clinical evaluation, myeloperoxidase (MPO) assay, neutrophil staining, western blot and quantitative polymerase chain reaction (qRT-PCR) were used to assess the effect of wedelolactone on *A. fumigatus* infection. Therapeutic effect of natamycin treatment with or without wedelolactone was measured via slit lamp microscopy.
Results: We confirmed that wedelolactone attenuated the infiltration of neutrophils and decreased MPO level at earlier time points in mice with *A. fumigatus* keratitis. Pre-treatment with wedelolactone decreased pro-inflammatory cytokine interleukin 1 beta (IL-1β) maturation by inhibiting caspase-1 activity. Combined with natamycin, wedelolactone protected corneal transparency in mouse with fungal keratitis.
Conclusion: Present findings indicated that wedelolactone reduced host immune responses by attenuating neutrophil recruitment and IL-1β maturation in *Aspergillus fumigatus* keratitis. Wedelolactone combined with an antifungal medicine could be a potential therapy for reducing lesion severity in fungal keratitis.

1. Introduction

Fungal keratitis, mostly caused by *Fusarium solani* and *Aspergillus fumigatus*, is one of the major causes of infection induced blindness in developing countries [1–3]. Although new agents including voriconazole and natamycin have already come into clinical practice, fungal keratitis still remains a challenge to oculist for its generally elusive diagnosis and extensive damage to corneal transparency [4,5].

Pathogenic fungi activate pattern recognition receptors (PRRs) and induce chemokines resulting in neutrophil recruitment into the corneal stroma [6]. Neutrophils, although essential for combating the fungal infection, can cause irreversible corneal damage resulting in loss of transparency which is vital to the function of the cornea. Thus, while it is crucial to annihilate corneal pathogenic fungi, it is also important to attenuate neutrophil infiltration to maintain corneal clarity [7]. This dual strategy may help ophthalmologists to fight fungal keratitis.

To solve this problem, we focused on wedelolactone, which is a natural plant product conventionally used in Chinese medicine to treat inflammatory conditions. Wedelolactone has been reported to suppress lipopolysaccharides (LPS) induced tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase production through NF-κB pathway in RAW 264.7 cells [8]. In addition, cigarette smoke extract induced COX-2 and intercellular adhesion molecule 1 (ICAM-1) were remarkably reduced by wedelolactone via Nrf2 pathway in human bronchial epithelial cells [9]. Wedelolactone also inhibited monocyte chemoattractant protein-1 (MCP-1) expression via TLR2/NF-κB pathway in ocular surface epithelial cells [10].

We generated an in vivo mouse model and in vitro THP-1 macrophage model of *A. fumigates* keratitis to understand the role of wedelolactone in fungal keratitis. We confirmed that wedelolactone played a protective role to corneal transparency in mouse early fungal keratitis by attenuated neutrophil infiltration and decreased MPO level.

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Wedelolactone impaired interleukin 1 beta (IL-1β) maturation against *A. fumigatus* infection by inhibiting caspase-1 activity. Wedelolactone combined with an efficacious antifungal medicine may be a potential therapy to reduce lesion severity of fungal keratitis.

2. Materials and methods

2.1. Preparing Aspergillus fumigatus

A. fumigatus strain 3.0772 was purchased from China General Microbiological Culture Collection Center (Beijing, China) and cultured for 3–4 days on Sabouraud agar. Fresh conidia were scraped from the surface of Sabouraud agar, and quantified to a final concentration of 5×10^4 conidia/μL in PBS.

2.2. In vivo experiments

Eight weeks old C57BL/6 female mice were purchased from the Changzhou Cavens Laboratory (Jiangsu, China). Mice were treated and anaesthetized with 8% chloral hydrate before experiments 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),

To study the role of wedelolactone in *A. fumigatus* keratitis, eyes were randomly selected from each mouse received a subconjunctival injection (5 μL) of 30 μM of wedelolactone (SelleckChem) [11] or dimethyl sulfoxide (DMSO) on day 1 and 2 h before infection. *A. fumigatus* conidia (0.5×10^5/μL) were released into the corneal stroma with a 33-gauge Hamilton syringe, and eyes were examined by slit lamp microscopy for corneal opacification and ulceration at days 1 and 3 post infection. Ocular disease was graded using clinical scores ranging from 0 to 12 according to the scoring system by Wu et al [12]. Corneas were harvested for myeloperoxidase (MPO) assay, quantitative polymerase chain reaction (qRT-PCR) and western blot at day 1 after the mice models were established, and eyeballs were removed at day 1 for immunofluorescence staining.

To study the activation and role of caspase-1, *A. fumigatus* conidia (0.5×10^5/μL) were released into mice corneal stroma and the mice corneal stroma was harvested for western blot at 1/2, 1, 2, 3 and 5 days after the mice model was established. To study the therapeutic effect of wedelolactone in *A. fumigatus* keratitis, *A. fumigatus* conidia (0.5×10^5/μL) were released into mice corneal stroma. The infected eyes received a subconjunctival injection (5 μL) containing 30 μM of wedelolactone (SelleckChem) or DMSO as a control at day 1 after infection. Then natamycin eye drops (Natacyn, Alcon Laboratories) were used 4 times daily for 2 days. The effect was measured by slit lamp microscope.

2.3. In vitro experiments

THP-1 cells were purchased from China Center for Type Culture Collection (Wuhan, China). After differentiation into macrophages by PMA treatment [13], cells were cultured in RPMI-1640 medium at a density of 1×10^6/mL and treated with wedelolactone at a final concentration of 10 μM. After 2 h, the cells were infected with conidia of *A. fumigatus* in 12-well or 6-well plates at a multiplicity of infection (MOI) of 1 for 4 h (qRT-PCR) and for 16 h (western blot).

2.4. Immunofluorescence

Neutrophil infiltration in *A. fumigatus* keratitis was observed by immunofluorescent staining of frozen sections of mouse eyes after embedding in Optimum cutting temperature (OCT) compound (Tissue-Tek) as described previously [13]. Sections were stained with 10 μg/mL rat anti-mouse Neutrophil Marker Antibody (Santa Cruz) followed by Alexa Fluor 488-conjugated goat anti-rat antibody (1:1000; Cell Signaling Technology).

2.5. MPO

MPO in corneal tissue was assessed as described previously [14]. The slope of the line was determined to assay relative units of MPO/cornea.

2.6. Quantitative polymerase chain reaction

Expression of IL-1β mRNA in mouse cornea and THP-1 macrophages was detected after infection as described previously [15]. The primer pairs used were as follows: m-IL-1β CGCAGCAGCACATCAACAAGAGC and TGTCCTCATCCTGGAAGGTCCACG, h-IL-1β GCTGATGGCCCTAAACAGATGAA and TCCATGGCCACAACAACTGAC, m-β-actin GATTACTGCTCTGGCTCCTAGC and GACTCATCGTACTCCTGCTTGC, h-β-actin TGGCACCCAGCACAATGAA and CTAAGTCATAGTCCGCCTAGAAGCA.

2.7. Western blotting

Infected corneas and THP-1 cells were analysed by western blotting as described previously [15]. Blots were stained for anti-dendritic cell-associated c-type lectin-1 (Dectin-1) (Cell Signaling Technology), anti-lectin-type oxidized LDL receptor 1 (LOX-1) (ProteinTech), anti-mIL-1β (R&D), anti-hIL-1β (Cell Signaling Technology), anti-caspase-1 (Novus) and anti-β-actin (Cell Signaling Technology). HRP-tagged secondary antibodies were purchased from Cell Signaling Technology.

2.8. Statistical analysis

An unpaired two-tailed Student's *t*-test was used to determine the statistical significance of the clinical score, MPO assays, qRT-PCR and western blotting. Data were analysed using GraphPad 5.0 software and considered significant at $P \leq 0.05$. Data were represented as mean ± SD.

3. Results

3.1. Response of wedelolactone pre-treatment in mice with A. fumigatus keratitis

Slit lamp imaging at days 1 (Fig. 1A) and 3 (Fig. 1C) post infection was performed to assess the disease response in corneas of control and wedelolactone pre-treated mice. Clinical scores were higher at day 1 (Fig. 1B; $P < 0.01$) and lower at day 3 (Fig. 1D; $P < 0.01$) in controls compared to wedelolactone pre-treated mice.

3.2. Wedelolactone pre-treatment attenuated neutrophil infiltration and MPO levels in mice with A. fumigatus keratitis

Neutrophil infiltration was presented by NIMP-R14 staining in control and wedelolactone pre-treated mice corneas at day 1 post infection (Fig. 2A). Compared to control mice, the attenuated presence of neutrophils in the corneas of wedelolactone pre-treated mice was confirmed by positive staining (green). MPO level in the corneas of wedelolactone pre-treated mice was significantly down-regulation (Fig. 2B; $P < 0.05$) at day 1 post-infection.

3.3. Wedelolactone suppressed IL-1β maturation in mice with A. fumigatus keratitis

Pre-treatment of wedelolactone down-regulated the transcript abundance as well as protein expression of IL-1β in mouse corneas infected by *A. fumigatus* (Fig. 3A and B; $P < 0.05$). Expression of Dectin-1, LOX-1 and pro-IL-1β was not affected by wedelolactone pre-treatment (Fig. 3B; $P > 0.05$) at day 1 post-infection.

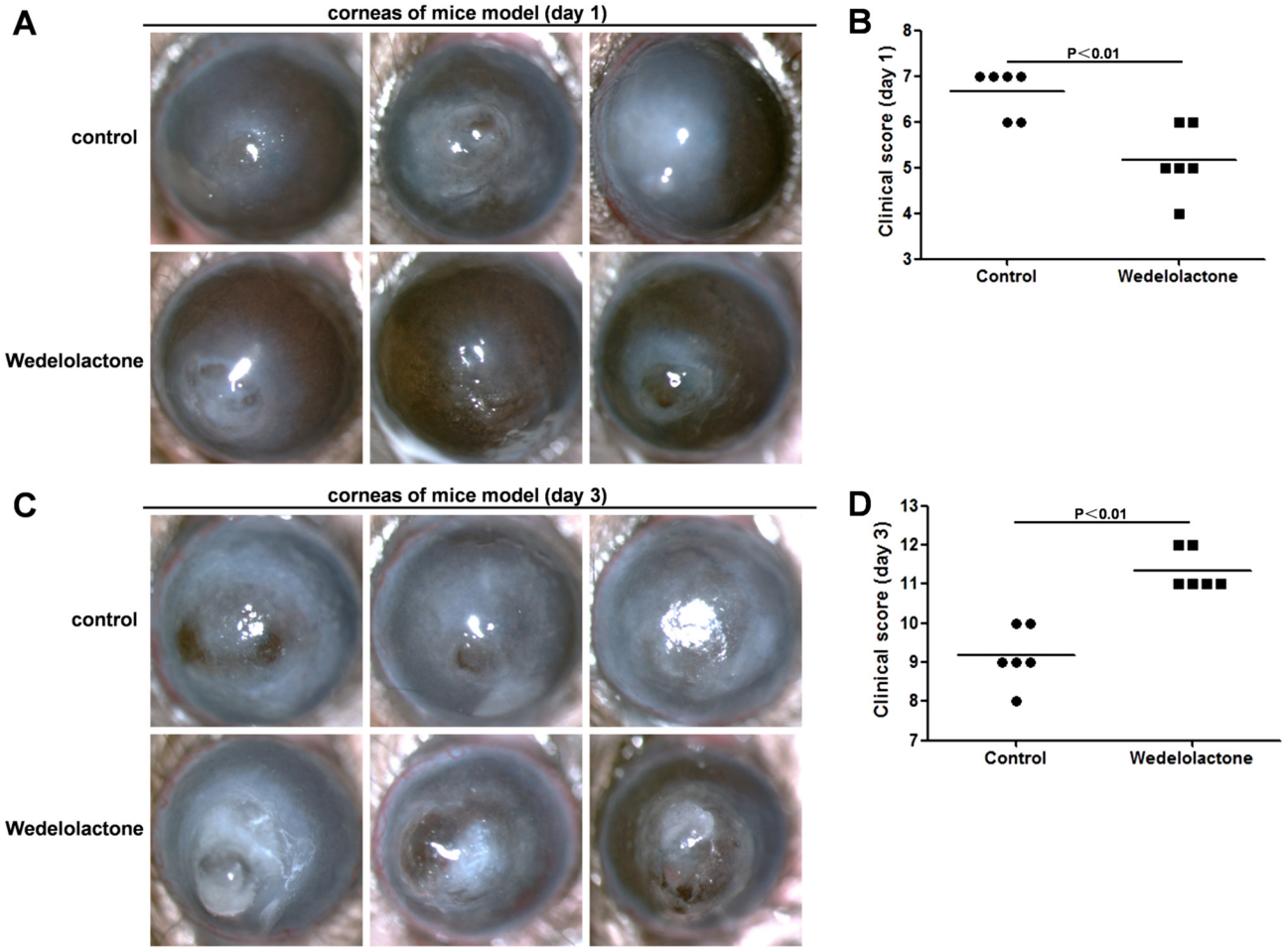

Fig. 1. Disease response with wedelolactone pre-treatment in mice with *A. fumigatus* keratitis. Slit lamp images at day 1 (A) and day 3 (C) post infection to illustrate the disease response in corneas of control and wedelolactone pre-treated mice. Clinical scores (n = 6/group) were used to represent the disease response. The score was higher at day 1 (B) and lower at day 3 (D) in controls compared with wedelolactone pre-treated mice.

3.4. Wedelolactone inhibited IL-1β maturation in human macrophages stimulated by A. fumigatus

Wedelolactone pre-treatment significantly down-regulated mRNA and protein expression of IL-1β in *A. fumigatus* stimulated THP-1 macrophages (Fig. 4A and B; $P < 0.05$ and 0.01, respectively). However, wedelolactone pre-treatment did not affect the expression of Dectin-1, LOX-1 and pro-IL-1β protein induced by 16 h stimulation with *A. fumigatus* (Fig. 4B; $P > 0.05$).

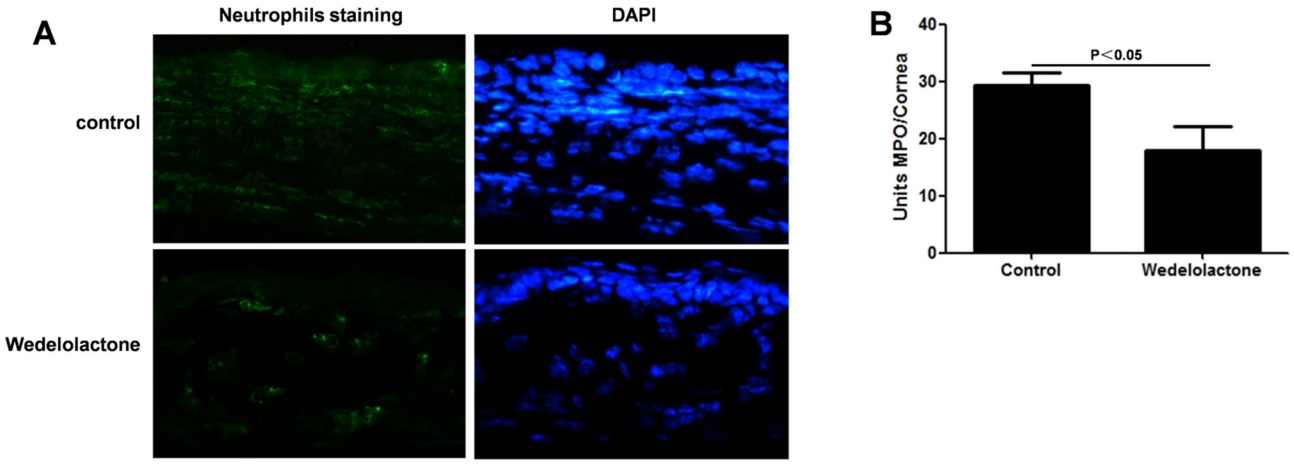

Fig. 2. Wedelolactone pre-treatment attenuated neutrophil infiltration and MPO levels in mice with *A. fumigatus* keratitis. (A) Positive staining (green) with a neutrophil marker antibody in the corneas of wedelolactone pre-treated mice indicated the decreased presence of neutrophils compared with control mice at day 1 post-infection (magnification ×400). (B) MPO level in corneas (n = 6/group) of wedelolactone pre-treated mice showed a significant down-regulation at day 1 post-infection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

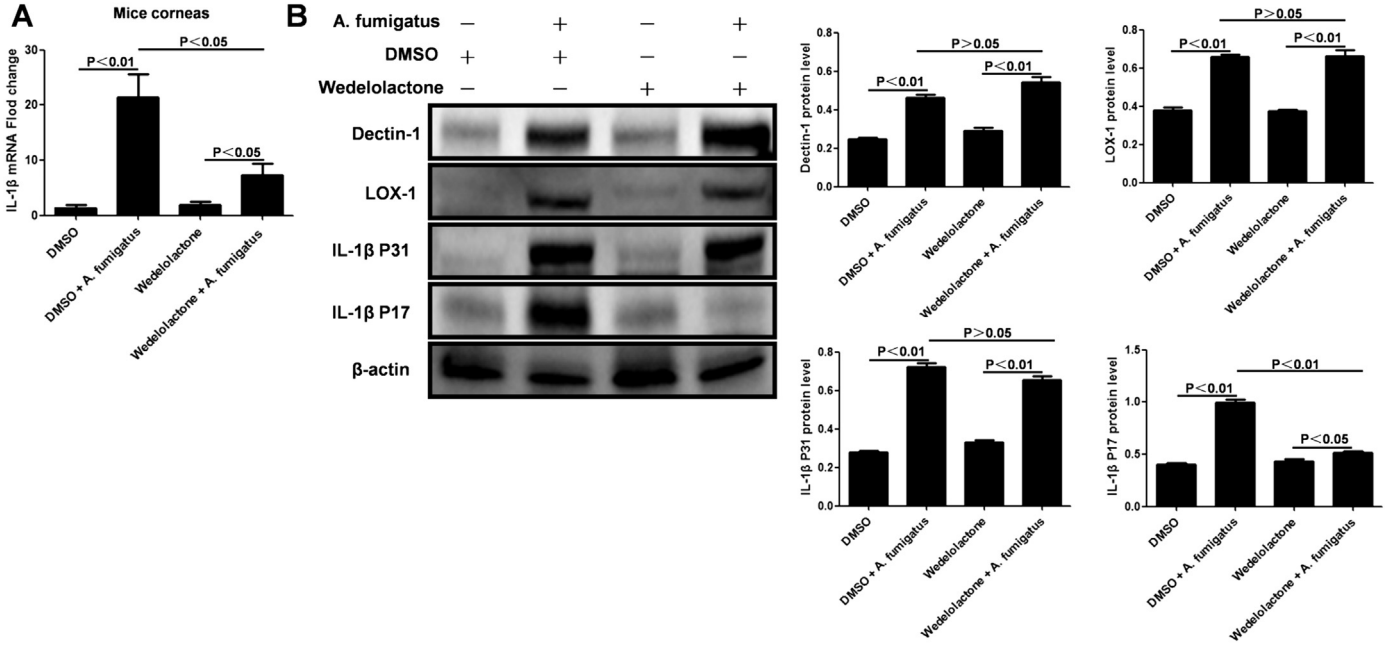

Fig. 3. Wedelolactone suppressed IL-1β maturation in mice *A. fumigatus* keratitis. (A) Pre-treatment with wedelolactone decreased transcript abundance of IL-1β in infected mouse corneas (n = 6/group) at day 1 post infection. (B) Dectin-1, LOX-1 and pro-IL-1β in corneas were not affected by wedelolactone pre-treatment at day 1 post-infection. Protein expression of mature-IL-1β was significantly lower in wedelolactone pre-treated mice.

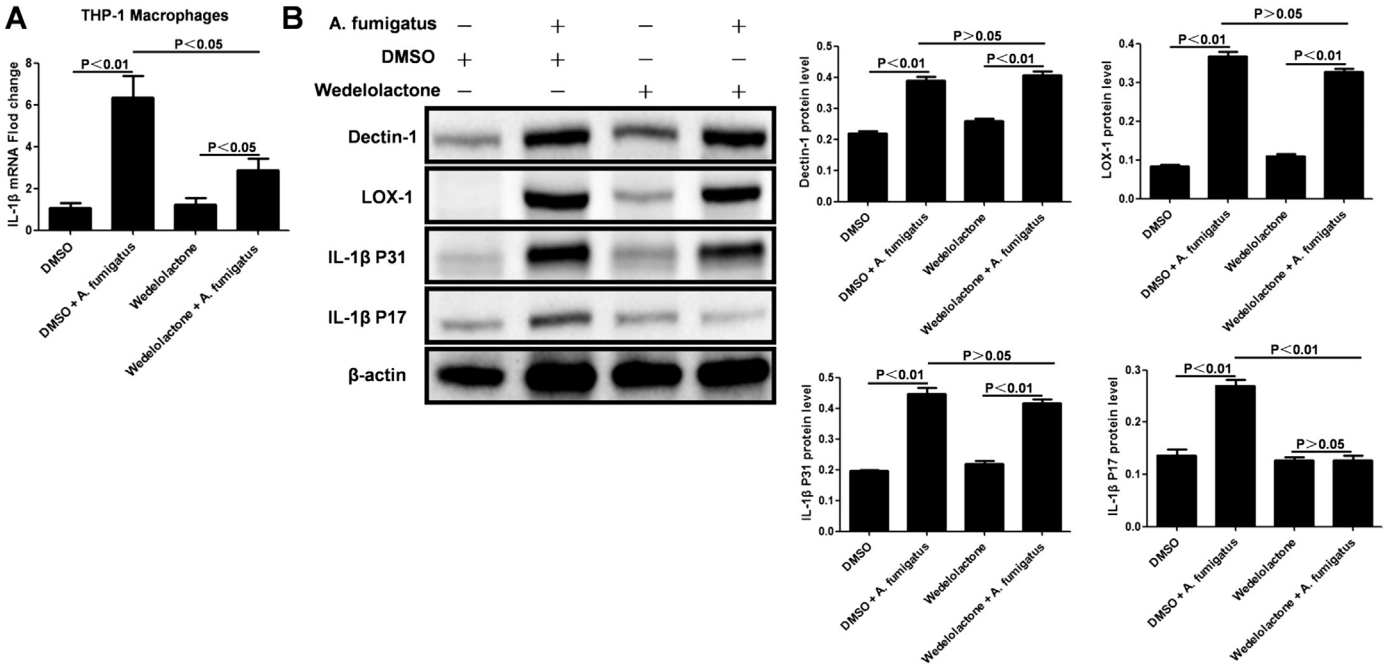

Fig. 4. Wedelolactone inhibited IL-1β maturation in human macrophages stimulated by *A. fumigatus*. (A) Pre-treatment with wedelolactone down-regulated mRNA expression of IL-1β in *A. fumigatus* stimulated THP-1 macrophages. (B) Pre-treatment with wedelolactone did not affect the expression of Dectin-1, LOX-1 and pro-IL-1β protein levels in THP-1 macrophages after 16 h stimulation with *A. fumigatus*. Expression of mature-IL-1β was significantly lower in wedelolactone pre-treated cells.

3.5. Wedelolactone regulated IL-1β maturation by inhibiting caspase-1 activity induced by A. fumigatus infection

Infected corneas collected at different time points (12 h to 5 days post infection) exhibited elevated expression of mature caspase-1 which was significantly reduced by wedelolactone pre-treatment at day 1 post infection (Fig. 5A and B; $P < 0.01$).

3.6. Wedelolactone in combination with natamycin contributed to the treatment of mice A. fumigatus keratitis

Slit lamp imaging at day 3 (Fig. 6A) post infection revealed that the disease response score was higher in only natamycin treated group compared with wedelolactone combined with natamycin treated mice (Fig. 6B; $P < 0.05$).

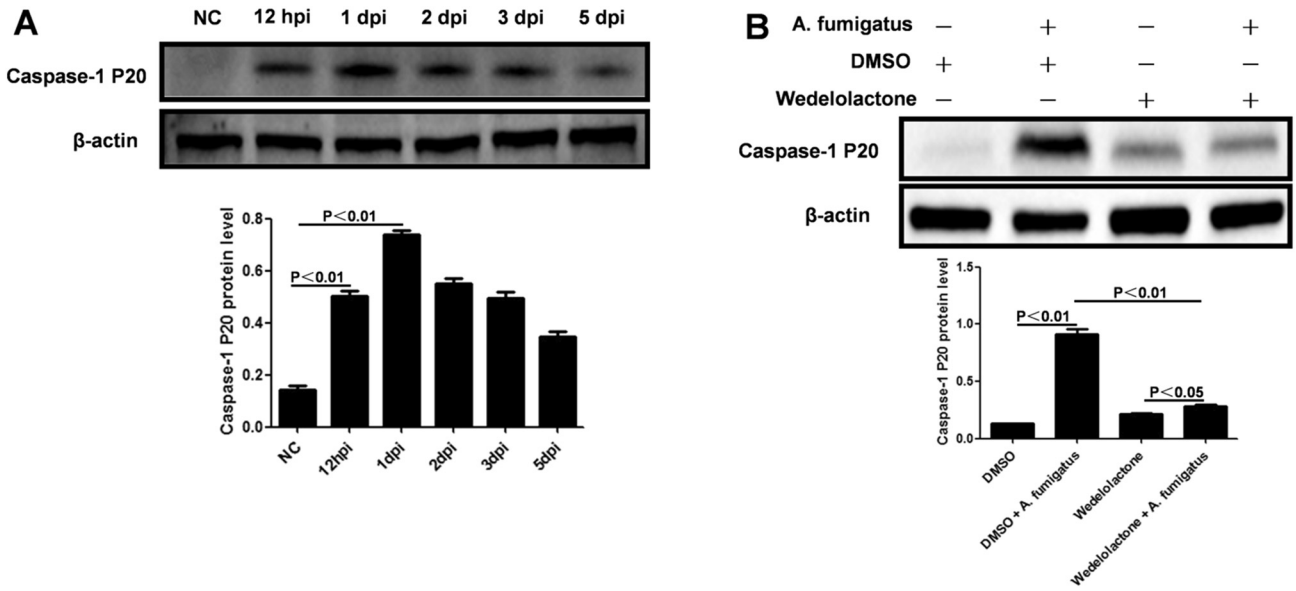

Fig. 5. Wedelolactone regulated IL-1β maturation by inhibiting caspase-1 activity induced by *A. fumigatus* infection. (A) Western analysis of mature-caspase-1 protein showed elevated expression in infected mice corneas (n = 6/group) 12 h to 5 days post infection. (B) Wedelolactone pre-treatment significantly lowered mature-caspase-1 expression in cornea (n = 6/group) at day 1 post infection.

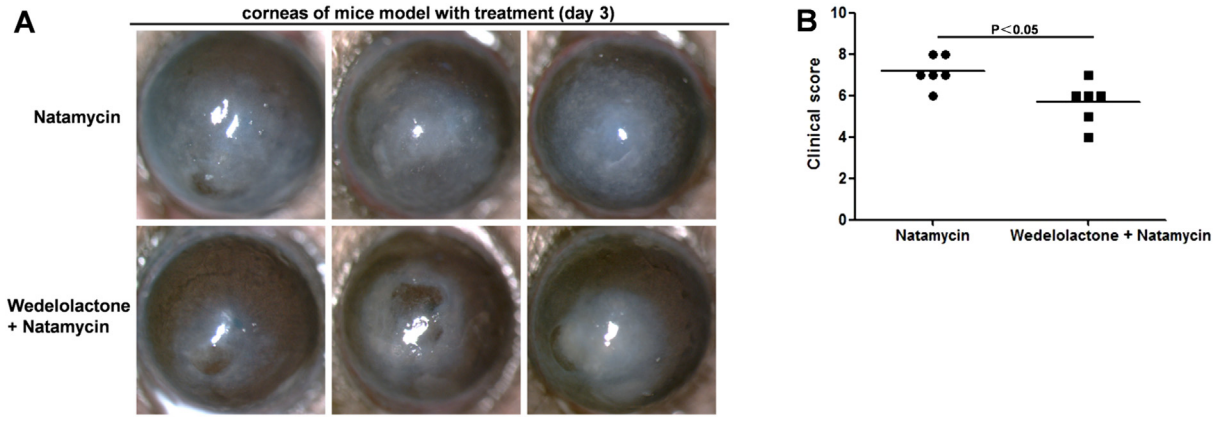

Fig. 6. Wedelolactone contributed to the treatment of mice *A. fumigatus* keratitis. (A) Slit lamp images of day 3 post infection cornea illustrating disease severity in mice treated with only natamycin (control group) and wedelolactone combined with natamycin (treatment group). (B) Clinical scores (n = 6/group) were higher at day 3 in natamycin treatment compared with wedelolactone combined with natamycin treated mice.

4. Discussion

Our findings indicated that wedelolactone may regulate key steps in inflammatory responses through decreasing neutrophil infiltration and IL-1β maturation in mice with fungal keratitis. This study demonstrated that wedelolactone might play a protective role in preliminary stages of *A. fumigatus* keratitis. The anti-inflammatory properties of wedelolactone have been reported in several studies. Ali et al. [16] identified that inflammatory markers, MPO and COX-2, and mast cell trafficking induced by ultra-violet B radiation were suppressed by wedelolactone through NF-κB pathway in the murine skin. Cuong et al. [17] identified that wedelolactone inhibited the up-regulation of TNF-α, IL-6, IL-12, superoxide generation, NADPH oxidase and the phosphorylation of p47phox induced by zymosan in murine bone marrow-derived macrophages. In a genome-wide transcriptome analysis, wedelolactone was reported to inhibit the phosphorylation of IKKα/β and production of IL-6, CXCL1 and CXCL8 in hormone-refractory prostate cancer [18]. These studies indicated that wedelolactone has the ability to suppress inflammatory responses in various diseases which is in line with the results of the present study.

Wedelolactone pre-treatment effectively suppressed the immune response and increased the clinical scores in early stages of fungal keratitis. Suppressed neutrophil infiltration and MPO activity in corneas correlated with these results [19,20], which could cause extensive damage to the corneas in the later stages in the absence of antifungal treatment. Luo et al. [21] reported that wedelolactone attenuated the recruitment of immune cells including neutrophils, macrophages, NK cells and NKT cells and activation of T-cell in immune-mediated liver injury. Morel et al. [22] identified that it can reduce asthma by decreasing immune cell recruitment and inhibiting IL-13, IL-4 and IL-5 production in bronchoalveolar lavage. Also, wedelolactone mitigated pathological colonic damage, reduced the recruitment of immune cells, and decreased the activity of MPO and alkaline phosphatase via MAPKs and NF-κB signaling pathways in murine colitis [23].

We observed that wedelolactone pre-treatment impaired the maturation of the pro-inflammatory IL-1β in mice corneas and THP-1 macrophages. IL-1β is one of the pivotal mediators of host immune response to inflammatory stimulation [24,25]. Consistent with our results, Shen et al. [26] showed that LPS-induced up-regulation of IL-1β, TNF-α and NO was inhibited by wedelolactone in a dose-dependent manner in human renal mesangial cells. Wedelolactone was reported to reduce IL-6, IL-1β and TNF-α expression and attenuate cell apoptosis in

acute liver injury [27].

Our results also showed that Dectin-1 and LOX-1, which are important antifungal PRRs [3,14], were not affected by wedelolactone pre-treatment. Both Dectin-1 and LOX-1 may not be involved in the effect of wedelolactone on IL-1β maturation. Furthermore, we found that wedelolactone down-regulated *A. fumigatus* induced IL-1β maturation by inhibiting caspase-1 activity. Activated caspase-1 cleaves pro-IL-1β to mature IL-1β in an inflammasome complex dependent manner. The mature-IL-1β is excreted from the cells to further induce inflammatory responses in neighboring cells [28,29]. Consistent with our results, Miao et al. [30] reported that wedelolactone regulated caspase-1 activation and IL-1β maturation in renal fibrosis.

In addition, our findings indicated that wedelolactone combined with natamycin played a protective role in rescuing corneal transparency in early stages of fungal keratitis. As an antifungal antibiotic, natamycin is used to treat fungal keratitis caused by *Candida*, *Aspergillus* and *Fusarium* by inhibiting amino acids and glucose transport across the fungal plasma membrane [31,32]. Although treatment with only wedelolactone improved clinical scores in the early stages, it caused extensive damages in the later stages of the disease. Wedelolactone combined with an antifungal medicine may provide a better strategy for combating pathogenic fungi and reducing neutrophil infiltration to preserve corneal transparency.

Taken together, our findings indicated that wedelolactone, a natural plant product used in traditional Chinese medicine, may suppress inflammation by inhibiting infiltration of neutrophils and IL-1β maturation in fungal keratitis. Wedelolactone combined with an antifungal drug may be an effective treatment to reduce lesion severity of fungal keratitis.

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