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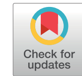
# The role of pirfenidone in alkali burn rat cornea



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ABSTRACT

To evaluate the effects of pirfenidone in the treatment of HUVEC using an in vitro model and on rat corneal wound healing, edema, cornea neovascularization (CNV) and inflammation after alkali burn in vivo model. In vitro, CCK-8 assay was used to detect the effect of pirfenidone on the viability of HUVECs. The effects of pirfenidone on migration and tube formation of HUVEC were evaluated by HUVEC cell wound closure and tube formation assay. In vivo, Eye drops containing pirfenidone or phosphate buffered saline (PBS) were administered to an alkali-burn-induced corneal inflammatory and neovascularization model four times daily. The clinical evaluations, including fluorescent staining and cornea edema, were performed on days 1, 4, 7 and 14 using slit lamp microscopy. Global specimens were collected on day 7 and processed for immunofluorescent staining Collagen IV, α-smooth muscle actin (α-SMA), vascular endothelial growth factor (VEGF), pigment epithelium derived factor (PEDF) and cluster of differentiation34 (CD34). The levels of α-SMA, VEGF, PEDF, CD34, CD31 and nuclear factor-kappa B (NF-κB) proteins in the corneas were determined by western blot. Pirfenidone affects HUVEC viability, migration and tube formation in a dose-dependent manner. High concentration of pirfenidone can inhibit HUVEC viability, migration and tube formation in vitro and reduce alkali burn rat cornea edema, promote corneal wound healing, inhibit CNV and inflammation after alkali burn in vivo. Pirfenidone promotes corneal wound healing, and inhibits cornea neovascularization and inflammation after alkali burn in vitro and in vivo. Pirfenidone may be the potential anti-inflammation agent for the clinical treatment of CNV.

## 1. Introduction

Alkali injury to the eye is one of the most common and devastating ophthalmic emergencies. The corneal alkali burn model is a well-established severe ocular surface disease model that causes corneal epithelial defects, prominent corneal acute inflammation, corneal neovascularization (CNV), and reduced corneal transparency [1]. It is widely used to study the mechanism and therapies of acute inflammation and angiogenesis, due to the easy local administration of medicine, well accessible position for observation, and the relatively immune-privileged status of the cornea [2]. Realizing the difficulty of treating corneal blindness once it has occurred, there is an enormous medical need to explore early and effective treatment options to enhance corneal wound healing, reduce corneal scarring, neovascularization and inflammation after alkali burn.

Pirfenidone (5-methyl-1-phenyl-2-[1*H*]-pyridone) is a new and broad-spectrum agent that has anti-fibrotic and anti-inflammatory effect in organs such as lung [3,4], liver [5], and kidney [6]. In a recent phase III multi-national clinical trial, pirfenidone has been shown to have beneficial effects for patients with various stages of idiopathic pulmonary fibrosis [7]. While the efficacy and safety of oral pirfenidone have been established in such specific diseases, limited data are available about the use of neovascularization and inflammation in alkali burns.

Pirfenidone was associated with improved conjunctiva bleb survival in rabbit glaucoma surgery model [8]. In addition, pirfenidone has been shown to inhibit expression of tissue inhibitors of metalloproteinases-1 and it has anti-fibrotic effects on orbital fibroblasts from patients with thyroid-associated ophthalmopathy [9]. Pirfenidone decreases collagen synthesis, prevents myofibroblast formation, improves corneal wound

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healing and prevents fibrosis following alkali burn [10]. Pirfenidone safely and effectively inhibits transforming growth factorβ1 (TGFβ1)-induced equine corneal fibrosis in vitro [11]. These findings suggest the potential therapeutic effects of pirfenidone for ocular diseases. Our study investigated the effect of pirfenidone in the treatment of HUVEC using an in vitro model and on rat corneal wound healing, edema, cornea neovascularization and inflammation after alkali burns in the in vivo model.

## 2. Materials and methods

### 2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from the Cell Line Bank of the Chinese Academy of Sciences (Shanghai, China). The endothelial cells were used after 2–6 passages.

### 2.2. Drug preparations

Pirfenidone was provided by Sigma-Aldrich Co. (St. Louis, MO). Pirfenidone was dissolved in phosphate buffered saline (PBS) to achieve a final concentration of 0, 50, 100, 200, 300, 500, 800 and 1000 μg/mL pirfenidone.

### 2.3. Cell counting kit-8 (CCK-8) assay

The respective HUVECs cell suspensions (100 μL containing $5 \times 10^3$ cells/well) were dispensed in triplicate into 96-well plates and incubated for 24 h. Pirfenidone was added to the medium at a concentration of 0, 50, 100, 200, 300, 500, 800 and 1000 μg/mL. After 72 h, 10 μL Cell CCK-8 solution (Dojindo, Kumamoto, Japan) was added to the wells, and the plates were incubated for 1 h. Absorbance (i.e., optical density [OD]) was read at 450 nm using a universal microplate reader (Bio-Tek, Winooski, VT, USA), and a graph of OD at 450 nm against concentration was plotted. Each mark represents the mean of the collected readings, and the procedure was repeated at least three times. Within 4 h, OD at 570 nm was determined using a microplate reader (ELX800, BIO-TEK Corporation, USA).

### 2.4. Wound closure assay for assessing migration

HUVECs cells migration was assessed using the wound-healing assay as previously described. HCE were seeded ($1 \times 10^5$ cells/well) in duplicate to 1% gelatin-coated 24-well plates (Corning, Schiphol, Netherlands). Cells were grown until confluence, and a scratch wound was applied in two perpendicular directions using a sterile pipet tip (200 μL yellow tip), thereby creating linear, cross-stripe scrapes that were 2 mm apart. Monolayers were washed with PBS to remove floating cells, the experimental medium (containing 0, 100, 500 and 1000 μg/mL pirfenidone) was added, and the cells were incubated for an additional 24 h. Cell migration to where the scrapes were introduced was photographed at different time points using an inverted microscope.

### 2.5. In vitro tube formation assay

HUVECs were serum-starved in EBM2 medium [0.1% fetal bovine serum (FBS) without growth factor] (Lonza, USA) for 12 h, and HUVECs were seeded to a density of 10,000 cells/well on growth factor-depleted Matrigel (BD Biosciences, NSW, Australia) in 24-well plates. Pirfenidone (0, 100, 500 and 1000 μg/mL) or the PBS (control supernatant) was added, and the results were quantified 6 h later. Microscopic fields containing the tube structures that formed in the gel were photographed at low magnification (10×). At least five fields in each well were examined. Before they were photographed, cells were fixed with 4% paraformaldehyde (PFA). Tube length was quantified using Image J software.

### 2.6. Alkali-burned rat cornea model

Alkali burns were applied to the Sprague Dawley rats (180–220 g; 2 months old; male; n = 26, Shanghai Shilaike Laboratory Animal Co, Ltd., Shanghai, China) as previously reported [7]. Briefly, anesthetized rats received topical administration of a drop of tetracaine. The alkali burn was induced by placing a 3.5 mm diameter round filter paper soaked with 1 N NaOH onto the center of the corneal surface for 30 s, followed by rinsing with 25 ml PBS.

Alkali burned animals were randomly divided into PBS group and pirfenidone group (26 rats per group). 10 μL PBS or 1000 μg/mL pirfenidone was topically administrated four times per day for 14 days. The administrated interval is 6 h (at am 6:00, am 12:00, pm 18:00, pm 24:00). Eyes were examined on day 1, 4, 7 and 14 by slit lamp microscopy to evaluate CNV, inflammation and damage. All animals were sacrificed on postoperative day 14, and the corneas samples were used for histological examination or protein extraction or stored at −80 °C.

Animal experiments were carefully performed in accordance with the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and all animal experimental procedures were approved by the Experimental Animal Committee of Xiamen University (approval ID: XMUMC2013-02-1).

### 2.7. Slit-lamp microscopic examination

Corneal epithelial changes were determined by 0.1% fluorescein sodium staining under cobalt blue light. Images were processed with Image Pro Plus V6.0 (Media Cybernetics, Silver Spring, MD, USA). CNV area (S) was quantified using the following formula: $S = C / 12 \times 3.1416 \times [r^2 - (r - I)2]$; where S is the area, C is time, I is the vessel radius, and r is the cornea radius [7]. The inflammatory index was evaluated based on various parameters as previously described, including ciliary hyperemia, peripheral and central corneal edema [11]. The classification criterion of corneal opacity was as follow: grade 0, completely transparent cornea; grade 1, minimal corneal opacity, but iris clearly visible; grade 2, mild corneal opacity, but iris vessels still visible; grade 3, moderate corneal opacity, pupil margin but not iris vessels visible; and grade 4, complete corneal opacity, pupil not visible.

### 2.8. Histology

Eye samples were fixed in 4% PFA in PBS overnight, dehydrated in a series of alcohol and embedded in paraffin. 5 μm thick sections were stained with hematoxylin and eosin (HE).

### 2.9. Immunofluorescent staining

Immunofluorescent staining was performed in cryosections (6 μm thick) of the eyeballs. Sections were fixed in acetone at −20 °C, blocked, and then incubated at 4 °C overnight with goat anti-Collagen IV antibody (cat. no. sc-29010, 1:100; Santa Cruz, USA), rabbit anti-α-smooth muscle actin (α-SMA) antibody (cat. no. sc-53142, 1:100; Santa Cruz, USA), rabbit anti-vascular endothelial growth factor (VEGF) antibody (cat. no. ab2349, 1:100; Abcam, UK), rabbit anti-pigment epithelium derived factor (PEDF) antibody (cat. no. sc-390172, 1:100; Santa Cruz, USA), anti-mouse cluster of differentiation34 (CD34) (cat. no. 50589M08H5, 1:500; Invitrogen, USA). After incubation with Alexa Fluor 488 donkey anti-goat, donkey anti-rabbit, donkey anti-mouse IgG (cat. no. W10810, 1:500; Invitrogen, Carlsbad, CA, USA), sections were counterstained with DAPI (cat. no. H-1500, Vector, Burlingame, CA, USA), mounted, and photographed using the Leica upright microscope (DM2500; Leica Microsystems, Wetzlar, Germany).

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