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Oxidative stress in corneal tissue in experimental keratitis due to *Aspergillus flavus*: Effect of topical voriconazole therapy

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ABSTRACT

The present study sought to determine whether topical voriconazole exerts antioxidative effects in experimental *Aspergillus flavus* keratitis. Three groups of five each young albino rabbits were used. Experimental keratitis was induced by application of *Aspergillus flavus* conidia to scarified cornea (right eye) of 10 rabbits (another 5 rabbits were negative controls [Group I]). Five days later, *A. flavus*-infected animals began receiving hourly topical saline (Group II) or voriconazole (10 mg/mL) (Group III). Twenty days post-inoculation, corneal lesions were graded; expression of interleukin-1beta (*IL-1β*) and tumor necrosis factor-α (*TNF-α*) genes, activities of three enzymatic antioxidants and reduced glutathione (GSH); malondialdehyde (MDA) levels were measured in corneal homogenates. Clinical, histopathological and microbiological scores suggested most severe keratitis in Group II and least severe in Group I rabbits. mRNA transcript levels of *IL-1β* and *TNF-α* were significantly higher in Group II than in Groups III and I rabbit corneal samples. Activities/levels of enzymatic antioxidants and of GSH were lowest in Group II and highest in Group I corneal samples, and MDA level was highest in Group II and least in Group I corneal samples. Corneal oxidative stress was more intense in *A. flavus*-challenged, saline-treated than in *A. flavus*-challenged, voriconazole-treated rabbits. Topical voriconazole notably reduced severity of keratitis and oxidative stress, although infection and inflammation did not completely resolve. Voriconazole thus exerts antioxidative effects, in addition to antifungal activity, in experimental *A. flavus* keratitis.

1. Introduction

Aspergillus flavus, an important species of the genus *Aspergillus*, has been reported as a common and virulent cause of fungal keratitis in different parts of the world (Thomas and Kaliamurthy, 2013; An et al., 2016). Septate hyphae sprout in linear and branching patterns that insinuate through the stromal lamellae in lesions of *Aspergillus* keratitis (Xie et al., 2008). Proteinases secreted by the infecting fungi may also contribute to the destruction of the corneal extracellular matrix (Gopinathan et al., 2001). Pro-inflammatory cytokines released from neutrophils infiltrating into a corneal lesion may promote the generation of reactive oxygen species (ROS) and increase oxidative damage in the inflamed cornea, thereby further contributing to the severity of keratitis (Leal et al., 2012). Superoxide dismutase (SOD) and glutathione peroxidase (Gpx) are reported to be the main antioxidant enzymes that protect the cornea from radical injury (Bilgihan et al., 2002).

The triazole antifungal agent, voriconazole, demonstrates good *in-vitro* activity against fungal species that are commonly implicated in keratitis, including *A. flavus* and *Fusarium solani* (Jurkunas et al., 2007). This broad-spectrum antifungal activity, coupled with good intraocular penetration following administration by various routes (Hariprasad et al., 2008), makes voriconazole ideal for use in the treatment of fungal keratitis. Overall, satisfactory results have been obtained when voriconazole is used to treat fungal keratitis (Thomas and Kaliamurthy, 2013), although topical voriconazole is reported to be less effective than natamycin for treatment of keratitis due to *Fusarium* species (Prajna et al., 2013).

Oxidative stress in corneal tissue has been implicated in the pathogenesis of *A. flavus* keratitis (Leema et al., 2013). Previous studies (clinical and experimental) that have evaluated the efficacy of voriconazole in treatment of fungal keratitis have tended to concentrate on clinical and related criteria. To our knowledge, the effect of voriconazole on manifestations of oxidative stress in infected corneal tissue, such as activities of enzymatic antioxidants (SOD, GPx, catalase[CAT]), levels of

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reduced glutathione (GSH) and malondialdehyde (MDA) and mRNA transcript levels of inflammatory cytokines (interleukin [*IL*]-*1β*, tumor necrosis factor alpha [*TNF-α*]) in fungal keratitis, has hitherto not been investigated. In the current study, these aspects have been evaluated in an experimental model of keratitis due to *A. flavus* in albino rabbits.

2. Materials and methods

2.1. Fungal isolate used to create experimental keratitis

2.1.1. Source and identification of fungal isolate

The strain of *A. flavus* used in the current study was isolated by an Ophthalmologist from corneal scrape material collected from a patient with ulcerative keratitis, who presented at Joseph Eye Hospital, Tiruchirappalli, India. A presumptive identification of the isolate as *A. flavus* was made by conventional microbiological techniques. DNA was extracted from the fungal genome and subjected to polymerase chain reaction (PCR)-based sequencing (EUROFINS, Bengaluru, India). PCR amplified a band with a sequence that had 99 % nucleotide homology with that of a reference standard strain of *Aspergillus flavus* ATHUM 5015 (GenBank accession number EU982036.1). The sequence data of the corneal isolate have been deposited on 4th November 2015 at GenBank as *Aspergillus flavus* strain PAT/LG, accession number KT999719.1.

2.1.2. Culture conditions

The *A. flavus* strain was first subcultured onto slopes of Sabouraud dextrose agar for cultivation of moulds (SDA, Himedia Laboratories, Mumbai, India) and incubated at 25–30 °C for 72 h for growth and sporulation. Conidia were harvested in physiological saline and suspensions of conidia were prepared to contain approximately 1×10^5 CFU/ml.

2.2. Experimental fungal keratitis

2.2.1. Experimental animals used

The animals in the present study were treated in accordance with institutional guidelines (Institutional Ethical Committee Approval Reference No. BDU/IAEC/2015/OE/10/dated 17th March 2015) and with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Research. Albino strains of young rabbits (either male or female), weighing between 2 and 3 kg body weight (b.w.) were divided into three groups, each group consisting of five animals:

1) Group I: Control (negative), received only saline
2) Group II: *A. flavus* challenged to induce keratitis
3) Group III: *A. flavus* + voriconazole (10 mg/ml)

Group I were negative controls (received only saline). Experimental *A. flavus* keratitis was induced in the right eye of rabbits in groups II and III; Group III rabbits were treated with topical voriconazole (10 mg/ml) and Group II rabbits were treated with topical saline. Animals were monitored daily, and severity of keratitis was scored at day 20 post-inoculation. Following animal sacrifice, excised corneas underwent investigations.

2.2.2. Induction of keratitis due to A. flavus in experimental animals

The animals were first immunosuppressed by intraperitoneal injections of cortisone (10 mg/kg b.w.) for three consecutive days prior to induction of infection. Anesthesia was achieved by an intramuscular injection of 25 mg/kg ketamine HCl, 2.5 mg/kg xylazine and topical proparacaine hydrochloride (Bilgihan et al., 2002). In each rabbit, corneal lesions were created in the right eye only, to avoid blinding the animal. A hypodermic needle was used to create a superficial wound of intersecting marks in a grid pattern on the cornea, as described earlier (Wu et al., 2003). Ten ml of the *A. flavus* conidial suspension were then applied topically to the scarified cornea of each animal in groups II and III; following application, the eyelids were kept closed for 15 min to facilitate contact between the fungal conidia and the scarified cornea, as a prelude to entry of fungi into the corneal stroma. Ten ml of 0.9% saline were applied topically in a similar manner to the scarified cornea of each animal in group I [control (negative), only saline].

2.2.3. Topical application of saline or voriconazole in experimental animals

Topical therapy with saline (Group II rabbits) or voriconazole (10 mg/ml) (Group III rabbits) was initiated when ulcerative keratitis first appeared (5 days after inoculation of *A. flavus*); the frequency of application was hourly until the keratitis definitely began to resolve, following which the frequency was reduced. Thrice-daily 1% cyclopentolate drops were also applied to achieve pupillary dilatation.

2.2.4. Scoring of clinical lesions in corneas of live rabbits

The rabbits in the three groups were monitored daily, and the severity of keratitis was scored at 20 days post-inoculation. A score of 0 (not present) to 4 (present and very marked) was assigned to each of three key parameters (the area of opacity, density of opacity and surface irregularity) to yield a possible total score of 0–12 (details tabulated in Table 1). A normal-appearing cornea received a score of 0 in each category. The severity of keratitis was categorized as mild (total score 1–3), moderate (4–8) and severe (total score ˃ 8) (Table 1). All animals were then sacrificed by cervical decapitation, and the right eyes were enucleated and carefully rinsed in sterile normal saline. From each enucleated eyeball, a 9 mm central corneal button was trephined, avoiding any trace of the corneoscleral limbus in the specimen. The button was dissected into equal bits for use in microbiological, histopathological and molecular investigations.

2.2.5. Recovery and identification of viable fungi and preparation of rabbit corneal tissue samples for analysis

One portion (20 mg) of each corneal button was placed in 1 ml of sterile saline in a small test tube and homogenized; the resulting homogenate was centrifuged at 12,000 xg for 15 min. The supernatant obtained was used for the antioxidant assays, GSH and MDA assays. The

Table 1
Scoring of clinical lesions in experimental *Aspergillus flavus* keratitis in albino rabbits.

Parameter evaluated	Grade of lesions			
	1	2	3	4
Area of corneal opacification (non-translucent zone)	≤ 25% of cornea involved	26%–50% of cornea involved	51%–75% of cornea involved	>75% of cornea involved
Density of visible corneal opacity	Barely detectable cloudy zone	Observable haze obscuring the iris	Marked opacity blurring the iris	An opaque leukoma obstructing view of the iris or pupil
Irregularity of corneal surface	Slightly distorted surface	Mild changes in corneal thickness	Moderate ulceration	Descemetocele or perforation present

A normal-appearing cornea received a score of 0 in each category.
The severity of keratitis was categorized as mild (total score 1–3), moderate (4–8) and severe (total score ˃ 8).

protein concentration in each fraction was determined by the method of Bradford (1976), using bovine serum albumin as a standard, while the deposited material was spread-plated on SDA plates in triplicate, which were then incubated at room temperature. After 48 h incubation, fungal colonies growing on the culture plates were counted. The fungi recovered in culture were confirmed to be *A. flavus* based on gross colony morphology and color, and also based on microscopic features (magnification X400) in lactophenol cotton blue-stained wet mounts (Hedayati et al., 2007).

2.3. Histopathological studies on rabbit corneal tissue

2.3.1. Preparation and staining of corneal tissue sections

Another portion of each corneal button was cut into pieces of the desired size and immediately fixed in Bouin's fluid at room temperature for 24 h, after which the tissue was transferred to 70% alcohol. The tissue was passed through several changes of 70% alcohol until the yellow color of the fixative had completely disappeared from the tissue, following which dehydration of the tissue was achieved by passing it through ascending grades of alcohol (30%, 50%, 70%, 90% and 100%). The tissue was then cleared in methyl salicylate, and embedded in paraffin wax at 57 °C. Sections (6–8 mm thickness) were cut from the paraffin-embedded tissue blocks using a rotary microtome (Leica, Nussloch, Germany); these sections were stained in Harris' haematoxylin and eosin and then washed in 90% alcohol for a few seconds. Gomori methenamine silver nitrate (GMS) staining was also performed (Pintozzi, 1978). The stained sections were dehydrated in 100% alcohol, cleared in xylene and mounted in DPX mountant, and then examined by bright-field microscopy (400x) (Carl Zeiss Axioskop 2 plus; Jena, Germany). Images were captured through a charge-coupled device camera in a computer and processed using Carl Zeiss Axio vision software (AxioVision 4.9.1 64bit software, Carl Zeiss Proprietary Limited, Sydney, New South Wales, Australia).

2.3.2. Scoring of histopathological lesions in rabbit corneal tissue sections

Histopathological readings were taken in a masked fashion and the lesions seen were scored. The parameters assessed were presence of fungal hyphae, presence of inflammatory cells and changes to the corneal stroma.

2.3.2.1. Scoring the presence of fungal hyphae. The scores were **0** = not present, **1** = 1 to 10 hyphae in 50 high power(x 400 magnification) fields (HPF), **2** = 1–10 hyphae in 10 HPF, and **3** = 1–10 hyphae per HPF.

2.3.2.2. Scoring the presence of inflammatory cells. The scores were **0** = not present, **1** = small numbers (background stroma and keratocyte nuclei seen clearly), **2** = moderate numbers (keratocyte nuclei obliterated but stroma visible), and **3** = many cells (complete obliteration of keratocyte nuclei and corneal stroma) per HPF.

2.3.2.3. Scoring changes to the corneal stroma. The scores were **0** = no alterations; **1** = mild alterations; **2** = a moderate degree of alterations; and **3** = marked destruction of corneal stromal structures.

Thus, the minimum histopathology score was 0 (seen in mock-infected rabbit corneas) and maximum score was 9 (seen in corneal tissue sections from some Group II (*A. flavus* treated/challenged to induce keratitis) rabbits.

2.4. RT-PCR analysis of inflammatory cytokines in rabbit corneal tissue homogenates

2.4.1. Extraction of total RNA

Total RNA was extracted from a portion of the corneal button by using TRIzol reagent (Sigma-Aldrich, St Louis, MO, USA). The purity and integrity of the isolated RNA were determined by spectrophotometry and agarose gel electrophoresis.

2.4.2. cDNA synthesis and PCR amplification

Total RNA was used as the template to generate first-strand cDNA in a 20 µl reaction volume as follows: 2 µg of total RNA were added to 1 µl of 10 mM dNTPs and 2 µl of 100 µM oligo dTs, made up to 10 µl with RNAse-free water, heated at 70 °C for 10 min and added to a reaction mix containing 2 µl of 10X reverse transcriptase buffer, 1 µl of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) enzyme (Promega, Madison, WI, USA) and RNAse-free water. The reaction mixture was incubated at 37 °C for 60 min and terminated at 95 °C for 5 min. PCR amplification of the cDNAs of the genes being studied, namely, *TNF-α* and *IL-1β* and of a 'housekeeping' gene (*β-actin*) (Table 2) was performed with a total reaction volume of 50 µl, consisting of PCR buffer (1X), 0.2 mM each of dATP, dGTP, dCTP and dTTP, 0.5 µM of each primer and 1.5 µl of Taq DNA polymerase (TAKARA Co. Ltd, Tokyo, Japan). After initial denaturation at 95 °C for 15 min, 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min and extension at 72 °C for 1 min) and a final extension at 72 °C for 10 min were performed in a thermocycler (Eppendorf, Hamburg, Germany). The concentration of the template and the number of cycles were optimized to ensure linearity of the response and to avoid saturation of the reaction.

2.4.3. Gel electrophoresis of PCR products and analysis of the bands obtained

On completion of the PCR reaction, 10 µl of each PCR product were subjected to electrophoresis in a 2% agarose gel containing ethidium bromide (0.5 µg/ml). Following electrophoresis, bands corresponding to transcripts of the study genes (*TNF-α* and *IL-1β*) and of the reference gene (β-actin) were first discerned by the naked eye, then photographed using a DS-34 type Polaroid camera and finally scanned by an imaging densitometer (Model GS-670, Bio- Rad, Hercules, CA, USA); the intensity of each band was analyzed by Quantity One software (Bio- Rad, Hercules, CA, USA). The relative expression level of each study gene was derived from the ratio of the densitometric reading of the study gene transcript to that of the *β-actin* transcript. Experiments were performed in triplicates.

2.5. Determination of antioxidant enzyme activities in rabbit corneal tissue homogenates

2.5.1. CAT

To measure CAT activity in the corneal homogenate sample,

Table 2
Sequences of primers used for amplification of target genes and expected size of the resulting PCR products in experimental keratitis due to *Aspergillus flavus* in albino rabbits.

S. No	Genes	Primer sequence	PCR product size	Annealing temperature (°C)
1	***TNF-α***	Forward primer 5′-GGGGGCCACCACGCTCTTCT-3′ Reverse primer 5′-AGGAGCACGTAGTCGGGG-3′	**336**	**52**
2	***IL-1β***	Forward primer 5′-GCCTCGTGCTGTCTGACCCA-3′ Reverse primer 5′-CAGGGTGGGTGTGCCGTCTT-3′	**198**	**50**
3	***β actin***	Forward primer 5′-GTGGCCGCTCTAGGCACCA-3′ Reverse primer 5′-CGGTTGGCCTTAGGGTTCAGGGGGG-3′	**108**	**51**

Abbreviations: ***TNF- α:* Tumor necrosis factor alpha**, *IL-1β:* Interleukin-1beta, *β*-actin was used as a "housekeeping" gene.

dichromatic acetic acid was reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchloric acid as an unstable intermediate; the green color that developed was read against a blank at 590 nm on a spectrophotometer (Spekol-1300, Analytik Jena, Munich, Germany) (Sinha, 1972). CAT activity was expressed as μmol of hydrogen peroxide [H_2O_2] consumed/min/mg protein.

2.5.2. SOD

SOD activity in the supernatant of the corneal tissue homogenate was determined by the pyrogallol autoxidation method, briefly, pH 8.2, 50 mM Tris-Cl buffer with 1 mM EDTA was used as reaction medium. corneal tissue homogenate containing 50–60 μg proteins was added to 0.2 mM pyrogallol (dissolved in pH 6.5, 50 mM PPB) to initiate the reaction, and the absorbance decrease of pyrogallol was monitored at 420 nm on a spectrophotometer (Spekol-1300, Analytik Jena,Munich, Germany). The percentage inhibition of pyrogallol autoxidation was calculated by the following formula:

% inhibition of pyrogallol autoxidation= [1-($\Delta A/\Delta A_{max}$)] x 100, where

ΔA= Absorbance change due to pyrogallol autoxidation in the sample reaction system.

ΔA_{max} = Absorbance change due to pyrogallol autoxidation in the control (without corneal tissue homogenate).

One unit of SOD activity was defined as the amount required for inhibiting pyrogallol autoxidation by 50% per min. (Marklund and Marklund, 1974). The SOD enzyme activity was expressed as units/mg protein.

2.5.3. GPx

GPx activity was measured by determining the rate of glutathione oxidation by H_2O_2, as catalysed by GPx present in the corneal tissue sample; the color that developed was read against a reagent blank at 412 nm on a spectrophotometer (Spekol-1300, Analytik Jena,Munich, Germany) (Rotruck et al., 1973). GPx activity was expressed as μg of reduced glutathione [GSH] consumed/min/mg protein.

2.5.4. Measurement of GSH levels in rabbit corneal tissue homogenates

GSH content in the corneal sample was measured by adding 4 ml of 0.3 M phosphate buffer (pH 8.0) and 0.5 ml of 0.04% (w/v) 5,5′-dithiobis (2-nitrobenzoic acid) to 1.0 ml of protein-free corneal sample; the absorbance of the resulting yellow color was read spectrophotometrically (Spekol-1300, Analytik Jena,Munich, Germany) at 412 nm. A standard graph was prepared using different concentrations of GSH in 0.3 ml of 5% trichloroacetic acid (TCA). The GSH content was calculated using this standard graph and expressed as nmol of GSH/mg protein (Moron et al., 1979). The GSH concentration was normalized to the protein concentration of the corneal tissue homogenate.

2.5.5. Determination of lipid peroxidation in rabbit corneal tissue homogenates

The concentration of MDA, a measure of lipid peroxidation, in the corneal homogenate sample was assayed in the form of thiobarbituric acid (TBA) - reacting substances. The test procedure yielded a pink color, the intensity of which was read spectrophotometrically (Spekol-1300, Analytik Jena,Munich, Germany) at 532 nm. The MDA was calculated with the help of a standard graph prepared by using different concentrations (1–10 nmol) of TMP (1,1,3,3-tetramethoxypropane) in 1 ml distilled water and expressed as nmol of MDA/mg protein. The MDA concentration was normalized to the protein concentration of the corneal tissue homogenate (Ohkawa et al., 1979).

2.6. Statistical analysis

Values are expressed as mean ± standard deviation for five animals in each group (n = 5). The statistical significance of differences between the mean values in the three groups was assessed by one-way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) for Windows (Version 16; SPSS, Inc, Chicago, IL, USA). If one-way ANOVA yielded significant results, post-hoc testing was performed for inter-group comparisons using Tukey's method. Differences were considered statistically significant when $P \leq 0.05$.

3. Results

3.1. Clinical scores of corneal lesions in rabbits of the different groups

With reference to corneal lesions in the rabbits (Fig. 1), the clinical score (8.5 ± 0.88) in Group II rabbits (*A. flavus* challenged to induce keratitis) was significantly higher ($P < 0.01$) than the score (3.76 ± 1.75) in Group III (*A. flavus* + voriconazole [10 mg/ml]) and the score (0.0) in Group I (negative control, only saline) rabbits (Table 3). The clinical score in Group III rabbits was also higher than that in Group I rabbits (Table 3).

3.2. Histopathological changes in rabbit corneal tissue sections

Sections from Group II rabbits (*A. flavus* challenged to induce keratitis) showed marked polymorphonuclear infiltration and stromal thickening, sections from Group III rabbits (*A. flavus* + voriconazole [10 mg/ml]) showed less polymorphonuclear infiltration and stromal thickening and sections from Group I (negative control, only saline) rabbits showed negligible infiltration (Fig. 2). In the corneal tissue sections, histopathological scores were higher ($P < 0.01$) in Group II (7.29 ± 0.08) than in Group III (2.66 ± 0.37) and fungal hyphae were more numerous in Group II than in Group III (Fig. 2; Table 3). Fungal hyphae, inflammatory cells and corneal stromal changes were not seen in Group I corneal sections (Fig. 2).

3.3. Recovery and identification of viable fungi from rabbit corneal tissue homogenates

Homogenized corneal bits (of uniform weight) from Group II rabbits (*A. flavus* challenged to induce keratitis) yielded 13.16 ± 1.83 *A. flavus* colonies in culture which was significantly higher than the number (2.83 ± 1.99) recovered from samples of Group III rabbits (*A. flavus* + voriconazole [10 mg/ml]) (Table 3). The microscopic and macroscopic morphologies of the strains recovered (progeny strains) were similar to that of the parent strain. *A. flavus* was not grown in culture of the corneal bits from Group I [negative control, only saline] rabbits (Table 3).

3.4. Measurement by RT-PCR of mRNA transcript levels of inflammatory cytokines in homogenates of rabbit corneal tissue

The mRNA transcript levels of the *IL-1β* (Fig. 3a and b) and *TNF-α* (Fig. 4a and b) genes were significantly higher in homogenates from Group II rabbits than from Group III and Group I rabbits.

3.5. Activities of enzymatic antioxidants and GSH and MDA levels in homogenates of rabbit corneal tissue

SOD (units/mg protein), CAT (μmol H_2O_2 utilized/min/mg protein) and Gpx (μmol GSH oxidized/min/mg protein) activities and GSH level (μmol/g tissue), were lower in corneal samples from Group II rabbits (*A. flavus* challenged to induce keratitis) (14.35 ± 1.35, 52.18 ± 5.64, 36.18 ± 0.22 and 48.19 ± 2.76, respectively) than the corresponding values (16.69 ± 2.41, 56.16 ± 4.20, 45.57 ± 4.93 and 55.51 ± 3.59, respectively) in samples from Group III (*A. flavus* + voriconazole [10 mg/ml]) rabbits; the differences were statistically significant ($P < 0.01$) for GPx and GSH (Table 4). SOD, CAT and Gpx activities and GSH level were significantly ($P < 0.05$) lower in Group III corneal samples than in Group I samples (Table 4).

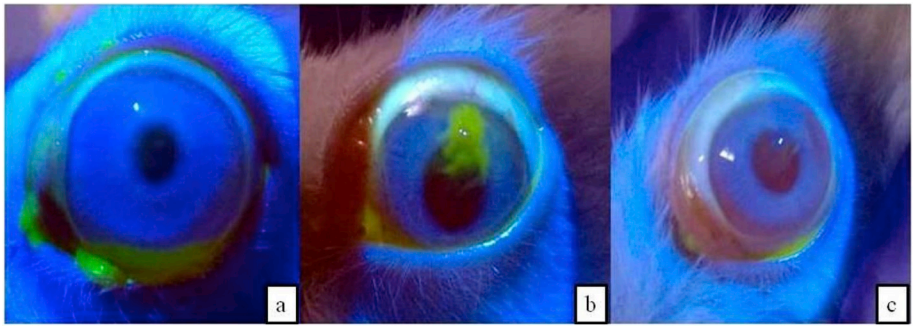

Fig. 1. Experimental keratitis due to *Aspergillus flavus* in rabbits.
Staining by fluorescein green highlights the ulcerated area of the corneal epithelium.
a) Group I: Control (negative), only saline:
b) Group II: *A. flavus* treated/challenged to induce keratitis.
c) Group III: *A. flavus* + voriconazole (10 mg/ml). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Mean clinical (corneal) and histopathological scores and mean number of fungal colonies recovered from corneas in experimental keratitis due to *Aspergillus flavus* in albino rabbits. All values are expressed as mean ± standard deviation of readings for 5 rabbits in each group (n = 5).

Group No.	Rabbits	Mean clinical score[c] for corneas in the group	Mean histopathological scores[d] for corneas in the group	Mean number of fungal colonies[e] recovered from corneas in the group
I	Negative control, only saline	0.00	0.00	0.00
II	*A. flavus* challenged to induce keratitis	8.5 ± 0.88^{a}	7.29 ± 0.08^{a}	13.16 ± 1.83^{a}
III	*A. flavus* + voriconazole (10 mg/ml)	$3.76 \pm 1.75^{a,b}$	$2.66 \pm 0.37^{a,b}$	$2.83 \pm 1.99^{a,b}$

[a]Statistically significant difference ($P < 0.05$) when compared with group I values.
[b]Statistically significant difference ($P < 0.05$) when compared with group II values.
[c] **Clinical scoring** performed at 20 days post-inoculation.Scores based on area and density of corneal opacity and on surface irregularity (details in Table 1) **Scores in these categories tallied for each eye to yield a total score ranging from 0 to 12.**
[d] **Histopathological scoring** performed in a masked fashion by two of the investigators on stained sections of excised corneal tissue obtained following animal sacrifice. Scores based on presence of fungal hyphae, presence of inflammatory cells and changes to corneal stroma. **a)** *Presence of fungal hyphae* **0** = fungal hyphae not present (in 50 high power microscope fields[HPF])(x400 magnification); **1** = 1 to 10 fungal hyphae present in 50 HPF; **2** = 1–10 hyphae in 10 HPF; **3** = 1–10 hyphae per HPF. b) *Presence of inflammatory cells* 0 = not present in 50 HPF; 1 = small numbers (background stroma and keratocyte nuclei seen clearly) per HPF; 2 = moderate numbers (keratocyte nuclei obliterated but stroma visible) per HPF; 3 = many cells (complete obliteration of keratocyte nuclei & corneal stroma)/HPF. c) *Changes to corneal stroma* 0 = no alterations; 1 = mild alterations; 2 = moderate degree of alterations; 3 = marked destruction of corneal stromal structures.Minimum histopathology score = 0 (seen in mock-infected negative control rabbit corneas) Maximum score= 9 (seen in some Group II [*A. flavus* treated/challenged to induce keratitis] rabbit corneas).
[e] Mean number of fungal colonies recovered after 48 h incubation on Sabouraud glucose-neopeptone agar plates that had been inoculated with the rabbit corneal tissue bits (20 mg) (mean number of colonies recovered from 5 corneas in each group [three plates used for each cornea]).

The MDA level (nanomoles/g tissue) in corneal homogenate samples from Group II rabbits (78.54 ± 0.04) was significantly higher ($P < 0.01$) than in Group III (70.14 ± 0.12) and Group I (67.24 ± 1.77) rabbits; similarly, there was a significant difference between MDA levels in Group I and Group III samples (Table 4).

4. Discussion

Pro-inflammatory cytokines released from neutrophils infiltrating a corneal lesion enhance ROS release and oxidative damage in an inflamed cornea, thereby further contributing to the severity of fungal keratitis (Leal et al., 2012). In experimental *A. flavus* keratitis in Wistar rats (Leema et al., 2013), significantly higher MDA and GSH levels, antioxidant enzyme activities and *IL-1β* and *TNF-α* gene expression were noted in corneas of aflatoxigenic *A. flavus*- challenged rats than in corneas of non-aflatoxigenic *A. flavus* challenged rats; clinical and histological scores were also higher in the first group than in the second group. It was concluded that aflatoxigenicity is associated with more intense oxidative stress in experimental *A. flavus* keratitis (Leema et al., 2013).

Voriconazole has proven effective in treatment of ocular fungal infections, particularly fungal keratitis (Thomas and Kaliamurthy, 2013). Voriconazole also possesses anti-inflammatory activity, although this is less than that exhibited by ketoconazole and itraconazole (Steel et al., 2008). Based on the good results obtained when voriconazole was used to treat endophthalmitis due to *A. fumigatus* in an animal model, Xiang-Gen et al. (2011) opined that voriconazole acted by rapidly reducing inflammation, preventing tissue destruction, decreasing fungal growth in the tissues, and by eliminating recurrences. In addition, Tu et al. (2010) reported that the introduction of voriconazole induced a rapid reduction in inflammation and allowed for a discontinuation of chronic topical immunosuppressant use, with a stable improvement of vision and comfort, in chronic *Acanthamoeba* stromal keratitis.

Several studies have been carried out using animal models of aspergillosis to study antifungal drug efficacy (Abdulaziz et al., 2012; Ernst et al., 2005; Hope et al., 2007; Paulussen et al., 2014; Steinbach et al., 2004). Since these studies have used different models and modalities of immunosuppression, it is important to exercise caution in interpretation of the results obtained. The same drug may act differently against the same organism when used in different animal models of aspergillosis. We observed that voriconazole significantly reduced clinical, histopathological and microbiological scores (Figs. 1 and 2, Table 3) in corneas/corneal tissue sections/corneal tissue homogenates of rabbits (Group III) that had been challenged with *A. flavus* and treated with voriconazole, suggesting that topical voriconazole therapy was efficacious in this experimental model of *A. flavus* keratitis. The better bioavailability of topical and oral voriconazole, and the fewer toxic side-effects reported, including photopsia, skin rash, and elevated liver functions, has made voriconazole the therapy of choice in *Aspergillus* infections (Pearson et al., 2003). In the present study, 15 days of voriconazole therapy appeared to have eliminated the infecting *A. flavus* strain to a great extent, but not completely. A longer duration of therapy might have resulted in complete resolution of the clinical and histopathological manifestations of keratitis, and complete eradication of the

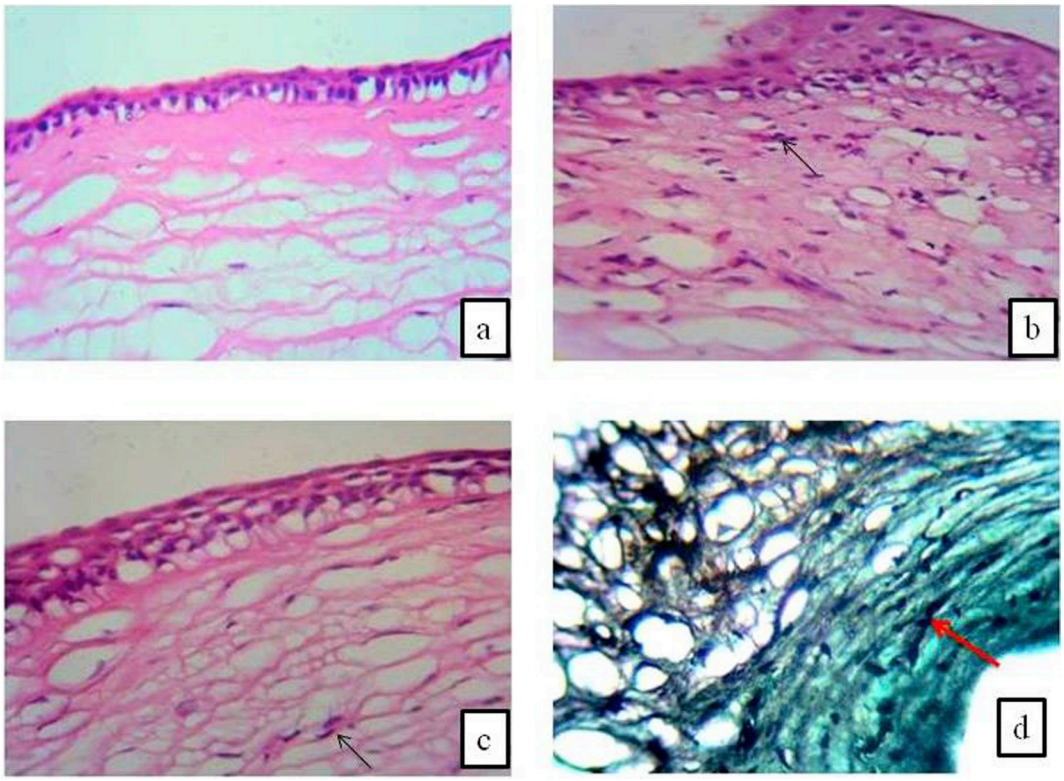

Fig. 2. Histopathology of experimental *Aspergillus flavus* keratitis in rabbits.
Corneas of immunosuppressed albino rabbits were mock-infected with saline (negative [non-infected] control) or challenged with 1×10^5 CFU/ml of conidia from a clinical isolate of *Aspergillus flavus* to create experimental keratitis. The animals were sacrificed after 20 days and the right eyeball was removed. From the right eye, the cornea was excised, fixed in formalin, embedded in paraffin, and sectioned (thickness of 10 μm). Sections were stained with haematoxylin-eosin and examined at 200 X and 400 X magnifications.
(a) Group I; no evidence of polymorphonuclear infiltration or stromal thickening; histopathology score= 0.00.
(b) Group II; marked polymorphonuclear infiltration (black arrow) and stromal thickening noted; histopathology score= 7.29 ± 0.08.
(c) Group III: a moderate degree of polymorphonuclear infiltration (black arrow) and stromal thickening noted; histopathology score= 2.66 ± 0.37.
(a, b, c) Sections of the cornea stained with haematoxylin eosin. (X400 magnification).
(d) Section of corneal tissue from Group II rabbits; fungal hyphae seen to penetrate stromal layers (red arrow) (**Gomori methanamine silver**, x400 magnification). Groups of rabbit corneas:1) Group I: It is control (negative), only saline: 2) Group II: *A. flavus* treated/challenged to induce keratitis 3) Group III: *A. flavus* + voriconazole (10 mg/ml). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

infecting fungus.

Keratitis represents a manifestation of corneal inflammation (Leck et al., 2002), which occurs as a defense mechanism to localize and eliminate an injurious agent and to remove damaged tissue components to facilitate healing. Hong et al. (2001) found that after corneal epithelial injury, pro-inflammatory chemokines were produced by keratocytes, therein triggering the influx of inflammatory cells into the corneal stroma. In the present investigation, mRNA transcript levels of *IL-1β* and *TNF-α* were higher in corneal homogenates from *A. flavus*-challenged rabbits with keratitis (Group II) than those in corneal homogenates from *A. flavus* + voriconazole (10 mg/ml)-treated (Group III) rabbits (Fig. 3a and b, Fig. 4a and b). In the negative control rabbit (Group I) corneal homogenates, the mRNA transcript levels of *IL-1β* and *TNF-α* were significantly lower than those noted in corneal homogenates from Group II and Group III rabbits (Fig. 3a and b, Fig. 4a and b).

Yamagami et al. (2003) opined that the inflamed cornea synthesizes the representative pro-inflammatory cytokines, *IL-1β* and *TNF-α*. Such a process possibly resulted in up-regulation of the *IL-1β* and *TNF-α* genes in the current study, as manifested by higher mRNA transcript levels of these genes in *A. flavus*-challenged, saline-treated (Group II) rabbit corneal homogenates than in the other groups. Increased levels of *IL-1β* at the ocular surface are reported to contribute to the epithelial damage observed in dry eye and in infectious eye diseases with an inflammatory component, by inducing expression of other pro-inflammatory cytokines in corneal epithelial cells and other ocular cells (Solomon et al., 2001; Narayanan et al., 2005, 2008). Such a process might have contributed to the observed greatest severity of keratitis in Group II rabbits in the present study. However, voriconazole treatment of *A. flavus*-challenged (Group III) rabbits resulted in significantly lower mRNA transcript levels of these pro-inflammatory cytokines in the cornea, resulting in a less severe keratitis in this group of rabbits.

The enzymatic antioxidants SOD, CAT, and GPx, which occur in all ocular tissues (Varma, 1991), are believed to protect the tissues by directly scavenging superoxide radicals and H_2O_2 by converting them to less reactive species. SOD catalyzes the dismutation of O_2^- to H_2O_2, and CAT and peroxidases reduce H_2O_2 to 2 H_2O. Thus, SOD and CAT serve, in tandem, as frontline antioxidant defenses (Scandalios, 2005). An enhanced intensity of oxidative stress in ulcerative keratitis is believed to disrupt the natural tissue balance of pro-oxidants versus antioxidants in favor of pro-oxidants (Čejková et al., 2004; Shoham et al., 2008). Lipid peroxidation is one of the most biologically-important free radical reactions (Finkel and Holbrook, 2000). MDA, an important aldehyde resulting from membrane lipid peroxidation, is often used as a marker of oxidative damage to lipids (Ohkawa et al., 1979).

In the present investigation, the activities of the enzymatic antioxidants, CAT, SOD and GPx, and the level of GSH (a non-enzymatic antioxidant) in homogenates of corneal tissue from Group II rabbits (*A. flavus*-challenged to induce keratitis) were significantly lower than those observed in corneal homogenates from Group I rabbits (negative controls) (Table 4); conversely, the MDA level was significantly higher

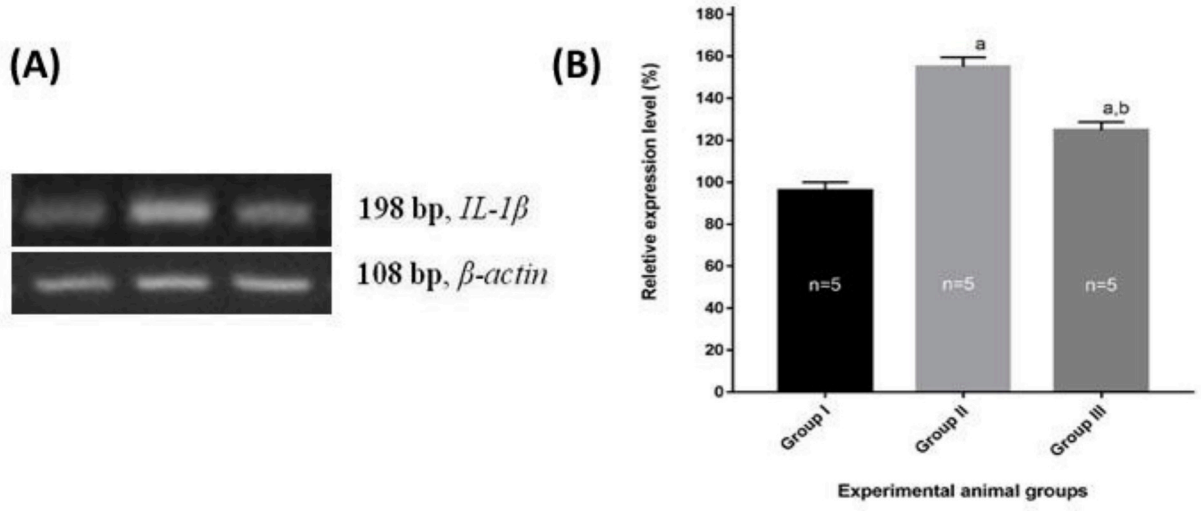

Fig. 3. mRNA transcript levels of interleukin-*1β* (*IL-1β)* gene in rabbit corneal tissue in experimental keratitis due to *Aspergillus flavus.* (a) Agarose gel (ethidium bromide-stained) results obtained following reverse transcription polymerase chain reaction for amplification of *IL-1β* gene in rabbit corneal tissue homogenates (**L1 -** 100 bp DNA ladder; **L2** - Group I corneas; **L3** - Group II corneas; **L4 -** Group III corneas).
(b) The results depicted are normalized to mRNA transcript levels of the control gene (β-actin).
Statistical analysis:
[a]Significantly different from Group I ($P < 0.05$) values;
[b]Significantly different from Group II ($P < 0.05$) values.
Groups of rabbit corneas:1) Group I: It is control (negative), only saline:2) Group II: *A. flavus* treated/challenged to induce keratitis 3) Group III*: A. flavus* + voriconazole (10 mg/ml).

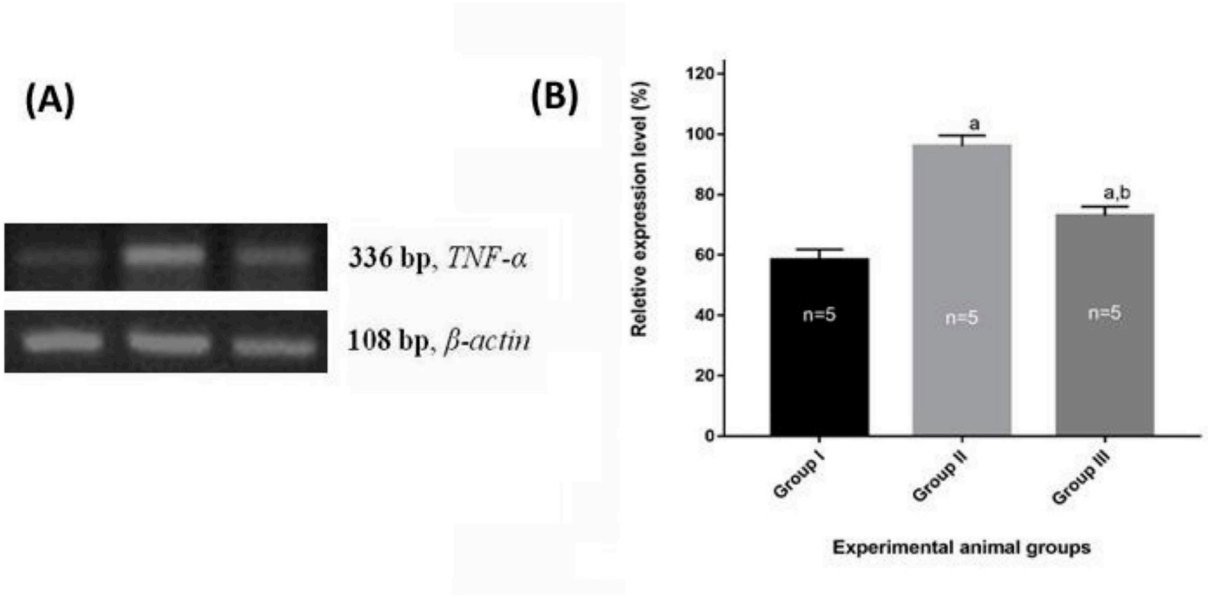

Fig. 4. mRNA transcript levels of tumor necrosis factor- alpha (*TNF-α*) gene in rabbit corneal tissue in experimental keratitis due to *Aspergillus flavus.*
(a) Agarose gel (ethidium bromide-stained) results obtained following reverse transcription polymerase chain reaction for amplification of *TNF-α* gene in rabbit corneal tissue homogenates (**L1 -** 100 bp DNA ladder; **L2** - Group I corneas; **L3** - Group II corneas; **L4 -** Group III corneas).
(b) The results depicted are normalized to mRNA transcript levels of the control gene (β-actin).
Statistical analysis:
[a]Significantly different from Group I ($P < 0.05$) values;
[b]Significantly different from Group II ($P < 0.05$) values.
Groups of rabbit corneas: 1) Group I: It is control (negative), only saline:2) Group II: *A. flavus* treated/challenged to induce keratitis
3) Group III: *A. flavus* + voriconazole (10 mg/ml).

in homogenates from Group II rabbits than that in Group I homogenates (Table 4). These changes possibly contributed to the severe inflammation (keratitis) seen in Group II rabbits (Fig. 1, Table 3). Interestingly, in corneal homogenates from *A. flavus*-challenged, voriconazole-treated (Group III) rabbits, the activities of CAT, SOD and GPx and level of GSH were higher (better), while the MDA level was significantly lower, than the values noted in corneal homogenates from Group II rabbits (the difference was statistically significant in the case of GPx and GSH), but

Table 4
Mean activities/levels of enzymatic and non-enzymatic antioxidants and of malondialdehyde in homogenates of corneal tissue from experimental keratitis due to *Aspergillus flavus* in albino rabbits.

Parameters tested	Mean values in group I rabbits (Negative control, only saline)	Mean values in group II rabbits (*A. flavus* -challenged to induce keratitis)	Mean values in group III rabbits (*A. flavus* + voriconazole (10 mg/ml))
SOD	21.37 ± 1.87	14.35 ± 1.35[a]	16.69 ± 2.41[a]
CAT	66.87 ± 2.66	52.18 ± 5.64[a]	56.16 ± 4.20[a]
GPX	55.49 ± 1.79	36.18 ± 0.22[a]	45.57 ± 4.93[ab]
GSH	61.18 ± 1.56	48.19 ± 2.76[a]	55.51 ± 3.59[ab]
MDA	67.24 ± 1.77	78.54 ± 0.04[a]	70.14 ± 0.12[ab]

Values represent the mean ± SD for observations made on five different rabbit corneas in each group (n = 5). Units: CAT= micromoles of hydrogen peroxide utilized per minute per milligram of protein; SOD= units per milligram of protein; Gpx= micromoles of GSH oxidized per minute per milligram of protein; MDA= micromole of MDA produced per milligram of protein.
Abbreviations: CAT= catalase, SOD= superoxide dismutase, Gpx= glutathione peroxidase, GSH= reduced glutathione, MDA= malondialdehyde.
Statistical analysis: one-way analysis of variance with post hoc testing (least significant difference).
[a]Statistically significant difference ($P < 0.01$) when compared with group I values.
[b]Statistically significant difference ($P < 0.05$) when compared with group II values.

still significantly different from the activities/levels in corneal samples from negative control rabbits (Table 4).

Thus, the gene expression results and activities/levels of enzymatic and non-enzymatic antioxidants and of MDA, taken in conjunction with the clinical, histopathological and microbiological scores, suggest that voriconazole therapy brought about a marked reduction in clinical and histopathological manifestations of experimental *A. flavus* keratitis, in the quantum of the infecting fungus and in the intensity of oxidative stress; however, voriconazole therapy did not result in complete eradication of the infecting fungus or in complete resolution of corneal inflammation/oxidative stress. Administration of voriconazole for a prolonged duration (for example, one month) may have brought about complete eradication of the infecting fungus and complete resolution of corneal inflammation; a disadvantage of this approach, however, is that side-effects of topical voriconazole therapy may also have become apparent. Oral voriconazole therapy could have been combined with topical voriconazole therapy to improve the outcomes. However, a recent study by Prajna et al. (2016) on a large series of cases concluded that there appeared to be no benefit to adding oral voriconazole to topical antifungal agents in the treatment of severe keratitis due to filamentous fungi. A potentially promising approach would be to use an antifungal with a non-steroidal anti-inflammatory agent to treat fungal keratitis; Fraser-Smith and Matthews (1987) concluded that ketorolac (a non-steroidal anti-inflammatory drug) appeared to suppress inflammation without exacerbating experimental *Candida albicans* ocular infection in rabbits. In future studies, we propose to explore the efficacy of topical voriconazole in combination with a topical antioxidant to treat experimental fungal keratitis.

5. Conclusion

The results of the present study suggest that corneal oxidative stress occurred in experimental keratitis due to *A. flavus*, since markers of oxidative stress (MDA levels, expression of *IL-1β* and *TNF-α* genes) were enhanced and activities/levels of enzymatic and non-enzymatic antioxidants were lowered, compared to levels in negative control corneas. The degree of oxidative stress appeared more intense in corneas of rabbits that had been challenged (infected) with *A. flavus* and treated with saline than that in corneas of rabbits that had been infected with *A. flavus* and treated with topical voriconazole. Topical voriconazole appeared to bring about a notable improvement in the severity of keratitis, although infection and inflammation appeared not to completely resolve within the therapeutic time frame. The therapeutic outcome of severe experimental fungal keratitis may be improved by combining topical antioxidant therapy (directed at reducing the intensity of corneal oxidative stress) with conventional antifungal drugs, but this hypothesis needs to be validated by additional studies.

Conflicts of interest

The authors declare that they have no conflict of interest.

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