· **Basic Research** ·

Effect of itraconazole on the cornea in a murine suture model and penetrating keratoplasty model

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Abstract

● **AIM: To investigate the anti-(lymph)angiogenic and/or anti-inflammatory effect of itraconazole in a corneal suture model and penetrating keratoplasty (PK) model.**

● **METHODS: Graft survival, corneal neovascularization, and corneal lymphangiogenesis were compared among itraconazole, amphotericin B, dexamethasone, phosphate buffered saline (PBS) and surgery-only groups following subconjunctival injection in mice that underwent PK and corneal suture. Immunohistochemical staining and analysis were performed in each group. Real-time polymerase chain reaction (RT-PCR) was performed to quantify the expression of inflammatory cytokines (TNF-alpha, IL-6) and vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGFR-2, and VEGFR-3.**

● **RESULTS: In the suture model, the itraconazole group showed less angiogenesis, less lymphangiogenesis, and less inflammatory infiltration than the PBS group (all *P*<0.05). The itraconazole group showed reduced expression of VEGF-A, VEGFR-2, TNF-alpha, IL-6 than the PBS group (all *P*<0.05). In PK model, the two-month graft survival rate was 28.57% in itraconazole group, 62.50% in dexamethasone group, 12.50% in PBS group, 0 in amphotericin B group and 0 in surgery-only group. Graft survival in the itraconazole group was higher than that in the amphotericin, PBS and surgery-only group (*P*=0.057, 0.096, 0.012, respectively). The itraconazole group showed less total angiogenesis and lymphangiogenesis than PBS group (all *P*<0.05).**

● **CONCLUSION: Itraconazole decrease neovascularization, lymphangiogenesis, and inflammation in both a corneal suture model and PK model. Itraconazole has anti-(lymph)-angiogenic and anti-inflammatory effects in addition to its intrinsic antifungal effect and is therefore an alternative treatment option in cases where steroids cannot be used.**

● **KEYWORDS**: itraconazole; amphotericin B; neovascularization; graft survival; lymphangiogenesis; dexamethasone

INTRODUCTION

The transplanted cornea is likely to be exposed to infectious organism, especially fungi, because frequent use of steroids to prevent rejection makes the cornea vulnerable to fungal infection[1]. Furthermore, previous ocular surgery, such as penetrating keratoplasty (PK) or keratotomy, is a predisposing factor for fungal keratitis[2-5].

Fungal keratitis is difficult to treat; even with antifungals, a considerable number of corneas need surgical intervention[1-5]. If there are signs of impending perforation irrespective of adequate medical treatment, therapeutic corneal transplantation should be performed[1]. Even after resolution of fungal keratitis, the cornea with profuse inflammatory neovascularization, lymphangiogenesis and opacity has difficulty to recover its clarity, resulting in decreased visual acuity.

Antifungals, especially after ocular surgery, should be sufficiently effective to eradicate the fungus, but also have low toxicity, and show anti-angiogenic and anti-inflammatory activity to maintain the integrity of the cornea. As anti-angiogenic and anti-inflammatory treatment, topical steroid can be used. However, topical steroid may not be considered until after clear clinical evidence of control of the infection.

We hypothesized that itraconazole which has broad antifungal activity can be safely used to decrease inflammation and angiogenesis from the beginning of disease course in fungal keratitis.

Here, to evaluate the anti-angiogenic and anti-inflammatory effects of itraconazole[6-9], we compared its effects in five groups: itraconazole, amphotericin B, dexamethasone,

phosphate-buffered saline (PBS) and surgery-only groups. We evaluated the anti-(lymph)angiogenic and anti-inflammatory effects of itraconazole in both a corneal suture model and a PK model.

SUBJECTS AND METHODS

The experiments were performed in accordance with the regulations of Association for Research in Vision and Ophthalmology (ARVO) and approval by the Institutional Animal Care and Use Committee (IACUC) of St. Vincent's Hospital.

Experimental Corneal Suture Model Seventy six eyes of 38 mice (BALB/c) were used in corneal suture model.

Corneal suture Two corneal sutures (10-0 nylon) were placed. To decrease inter-procedure variation, we made the sutures by one surgeon (Cho YK) in all mice of suture model. After making sutures in corneas of mice, we injected the four test materials (subconjunctival injection, 10 µL) individually into each appropriate group on the day of suture, and twice weekly thereafter until the six weeks post-suture; itraconazole (5 mg/mL, 10 eyes of 5 mice), amphotericin B (5 mg/mL, 10 eyes of 5 mice), glucocorticoid (dexamethasone sodium phosphate, 5 mg/mL, 10 eyes of 5 mice), and PBS (10 eyes of 5 mice).

For analysis of neovascularization (NV), lymphangiogenesis (LY) and CD11b+ cells infiltration, one control group was added: suture-only group (2 corneal sutures were made at 12 and 6 o'clock position between corneal center and limbus, no further subconjunctival injection was tried in this group). After harvesting, we compared NV and LY among the groups to assess the anti-angiogenic and anti-inflammatory effects of the treatments.

Grading of corneal neovascularization Microscopic images were captured using a surgical microscope (Leica EZ; Heerbrugg, Switzerland) at 3d, 2 and 4wk after suture placement and subconjunctival injection. Corneal NV was graded from 0 (no NV) to 5 (thick tortuous new vessel growth over the suture and toward the center of the cornea).

Immunohistochemical staining We measured inflammation, NV, and LY in the corneas. After harvesting the corneas, immunohistochemical staining for CD11b, CD31, and LYVE-1 was performed as previously described[10]. Seven of 10 eyes from each group were immunstained for CD31 and LYVE-1 for fluorescent microscopic examination, and the remaining 3 eyes of each group were immunostained for CD11b+ cells for confocal microscopic examination.

Fluorescent microscopic examination After immunohisto-chemical staining and flat mounting of the cornea, images of the corneal vasculature were captured and quantified using a camera attached to a fluorescent microscope (OLYMPUS BX51, Tokyo, Japan) and NIH Image J as previously described[10].

Confocal microscopic examination Two or three sites around sutures from each cornea were chosen in each group. A confocal microscope was used to quantify the area of inflammatory infiltration (LSM 700, Carl Zeiss, München-Hallbergmoos, Germany). Horizontal sections (objective magnification ×10) of 17 to 19 images were obtained from the top surface to the bottom of the cornea at 5 µm intervals and were stacked to create final image stacks. The percentage area of CD11b+ cell infiltration was analyzed in each image stack using the pixel area.

Quantitative real time polymerase chain reaction analysis of gene expression in the mouse cornea After making the suture model in corneas of mice, we injected the four test materials (subconjunctival injection, 10 µL) individually into each appropriate group on the day of suture, and twice weekly thereafter until the six weeks post-suture; itraconazole (5 mg/mL, 8 eyes of 4 mice), amphotericin B (5 mg/mL, 6 eyes of 3 mice), glucocorticoid (dexamethasone sodium phosphate, 5 mg/mL, 6 eyes of 3 mice), PBS (6 eyes of 3 mice) and suture-only group (4 eyes of 2 mice). After harvesting, the expression of VEGF-A, VEGF-C, VEGFR-2, VEGFR-3, TNF-alpha and IL-6 in each group were analyzed using real-time polymerase chain reaction (RT-PCR).

Total RNA was purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA synthesis and thermocycling was performed. We used published primer sequences for mouse GAPDH[10], mouse VEGF-A[11], mouse VEGF-C[11], mouse VEGFR-2[11], mouse VEGFR-3[11], mouse TNF-alpha[12], and mouse IL-6[13]. Each gene expression level was analyzed by the Ct method, using GAPDH expression as an internal control. The relative expression level of each sample is expressed as a fold change compared to the normal control (PBS).

Experimental Corneal Penetrating Keratoplasty Model Thirty eight eyes of 38 recipient mice (BALB/c) and 38 eyes of 19 donor mice (C57BL/6) were used in PK model. To decrease inter-procedure variation, we made total 8 corneal interrupted sutures for graft apposition by one surgeon (Cho YK) in all mice of PK model.

Corneal transplantation PK was performed as follows[14]: female mice (8 to 12 weeks old) of the BALB/c strain were graft recipients (8 eyes of 8 mice per group) and mice of the C57BL/6 strain (8 eyes of 4 mice per group) served as graft donors (The Koatech Laboratory, Pyeongtak, Korea). The recipient and donor mice were anesthetized as described previously[14-15]. The donor cornea was marked with a 2 mm trephine and was cut with Vannas scissors and placed in balanced salt solution (BSS® Alcon Laboratories, Inc., Fort Worth, USA). The donor graft was sutured into the recipient bed. After covering the eye with 0.5% ofloxacin ophthalmic ointment, the lid was sutured. All sutures were removed one week after transplantation.

After making PK model in corneas of mice, we injected the four test materials (subconjunctival injection, 10 μL) individually into each appropriate group on the day of PK, and weekly thereafter until the 8wk post-PK as follows; itraconazole (5 mg/mL, 8 eyes of 8 mice), amphotericin B (5 mg/mL, 8 eyes of 8 mice), glucocorticoid (dexamethasone sodium phosphate, 5 mg/mL, 8 eyes of 8 mice), PBS (8 eyes of 8 mice) and KP-only group (6 eyes of 6 mice, PK was done and no further subconjunctival injection was tried in this group).

Clinical evaluation of rejection The mice were examined and photographed weekly through postoperative week 8 using an operating microscope (OPMI 9-FC, Zeiss, Germany) and a camera (CVMV-K59, Ecwox, China). We evaluated clinical graft rejection according to the grading system as previously described[16]. The opacity grading (0 to 5) was made. Opacity grades 3 and above were considered as graft rejection.

Fluorescent microscopic examination After immunohistochemical staining, NV and LY were quantified as the same method used in corneal suture model.

Statistical Analysis Statistical analysis was performed using SPSS 11.5 (IBM, Armonk, NY, USA). Survival of the allografts was analyzed using Kaplan-Meier survival curves and the log-rank test. NV, LY, and inflammatory cell infiltration of the five groups were compared using an ANOVA with a post hoc test. A P value <0.05 was considered statistically significant.

RESULTS

Corneal Suture Model

Neovascularization Figure 1 shows grading of corneal NV and representative biomicroscopic pictures of five groups. In fluorescent microscopic examination, the total neovascularized area was smaller in the itraconazole group (5.64%±0.56%) and dexamethasone (3.39%±0.60%) than the PBS group (10.93%±0.69%) (ANOVA with post hoc test, P=0.039, <0.001, respectively). There was less NV in the dexamethasone group (3.39%±0.60%) than the amphotericin B (9.33%±1.36%) and PBS groups (ANOVA with post hoc test, P=0.014, 0.007, respectively). There was no difference of NV between PBS (10.93%±0.69%) and suture-only group (10.27%±1.50%) (Figure 2).

Lymphangiogenesis There was less total LY in the itraconazole (4.79%±0.83%) and the dexamethasone group (4.04%±0.07%) than the PBS (11.60%±0.53%) (ANOVA with post hoc test, P=0.032, 0.049, respectively). There was no difference of LY between PBS (11.60%±0.53%) and suture-only group (12.01%±2.01%) (Figure 3).

Inflammatory infiltration Inflammatory cell infiltration in the five groups is compared in Figure 4. Itraconazole (3.57%±0.19%) and dexamethasone (3.11%±1.11%) suppressed inflammatory infiltration compared to PBS (9.61%±2.01%) (ANOVA with post hoc test, P=0.049, 0.020,
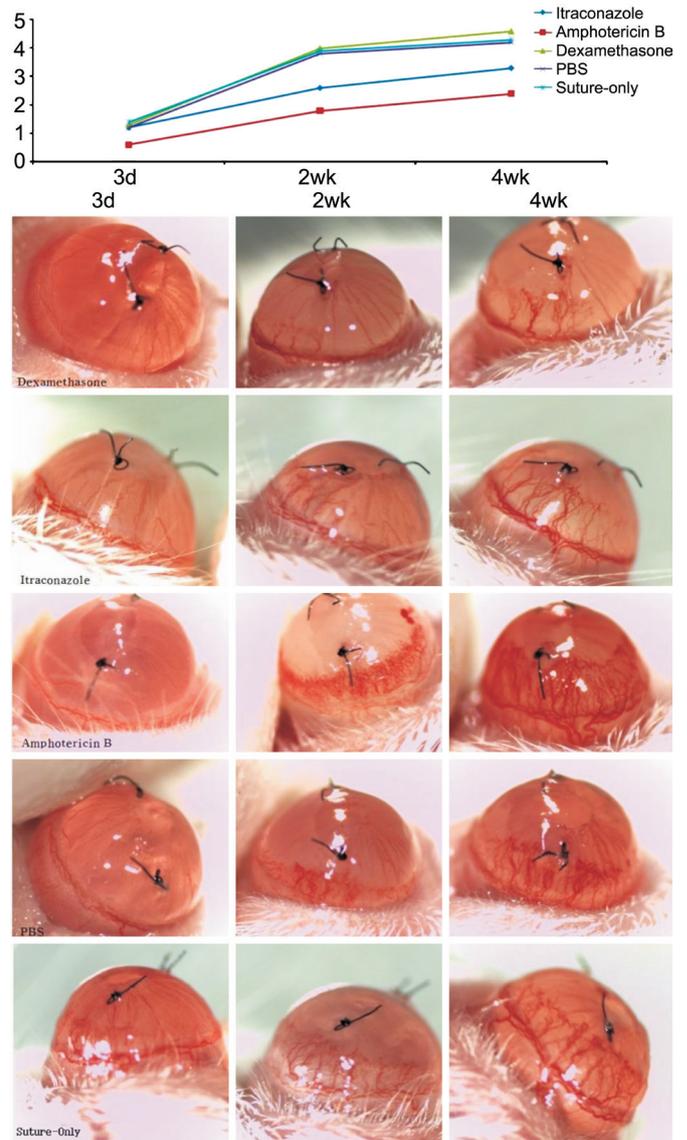

Figure 1 Grading of corneal neovascularization and biomicroscopic pictures (itraconazole, amphotericin B, dexamethasone, PBS, suture-only groups).

respectively). Itraconazole and dexamethasone suppressed inflammatory infiltration compared to suture-only group (11.73%±2.15%) (ANOVA with post hoc test, P=0.013, <0.01, respectively).

Real-time polymerase chain reaction Figure 5 shows the mRNA expression levels of VEGF-A, VEGF-C, TNF-alpha, IL-6, VEGFR-2, and VEGFR-3 in each group in suture model. mRNA expression ratios were normalized by GAPDH (PBS group=1.0). A P value represents the statistically significance compared with PBS group.

There was a significant difference in VEGF-A level among the itraconazole, amphotericin B, and dexamethasone groups (P<0.001). The itraconazole and dexamethasone group showed a significant reduction in VEGF-A expression compared to the PBS group. The itraconazole group had significantly reduced VEGF-A level compared to the PBS and amphotericin B groups (ANOVA with post hoc test, P<0.001, <0.001, respectively) (Figure 5A).
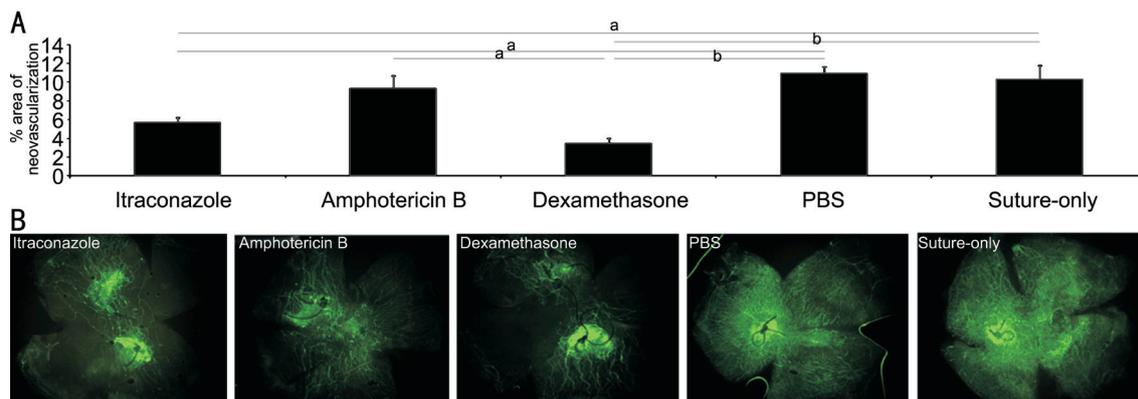
Figure 2 NV in suture model A: Comparison of NV in the corneal suture model among the five groups (itraconazole, amphotericin B, dexamethasone, PBS, suture-only groups) at 6wk post-sutures; B: Representative pictures. [a]$P<0.05$, [b]$P<0.01$.
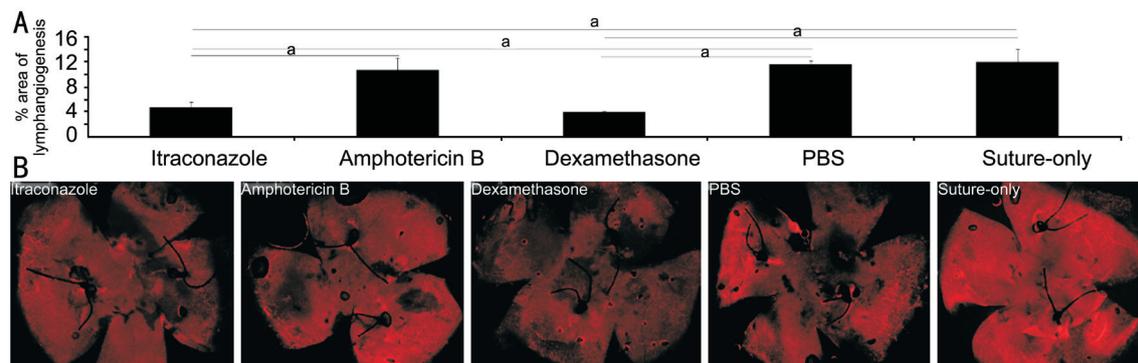

Figure 3 LY in suture model A: Comparison of LY in the corneal suture model among the five groups (itraconazole, amphotericin B, dexamethasone, PBS, suture-only groups) at 6wk post-sutures; B: Representative pictures. [a]$P<0.05$.
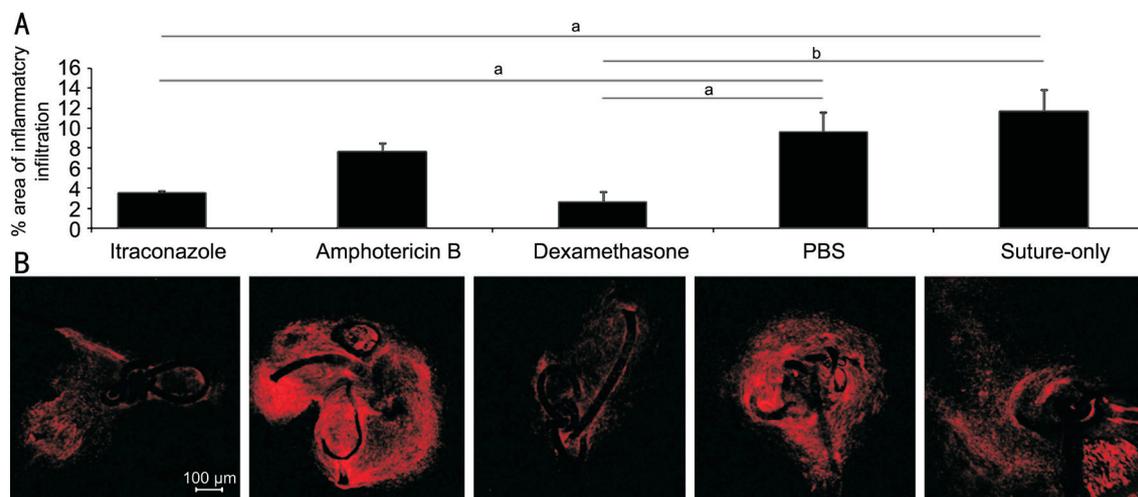

Figure 4 Inflammatory infiltration in suture model A: Comparison of inflammatory infiltration in the corneal suture model among the five groups (itraconazole, amphotericin B, dexamethasone, PBS, suture-only groups) at 6wk post-sutures; B: Representative pictures. [a]$P<0.05$, [b]$P<0.01$.

There was no difference in VEGF-C level among the three treatment groups ($P>0.05$). However, the itraconazole-treated group showed reduced VEGF-C expression compared to the PBS group (ANOVA with post hoc test, $P=0.098$) (Figure 5B). There was a significant difference in TNF-alpha level among the itraconazole, amphotericin B, and dexamethasone groups ($P<0.001$). The itraconazole group had a significantly lower level of TNF-alpha than the PBS and amphotericin B groups (ANOVA with post hoc test, $P<0.001$, <0.001, respectively) (Figure 5C). TNF-alpha expression was also significantly higher in the amphotericin B group than in the PBS group ($P<0.01$).

In comparison of IL-6, itraconazole and dexamethasone group showed significant reduction of IL-6 compared with PBS ($P<0.05$, <0.01, respectively, ANOVA with post hoc test). (Figure 5D).
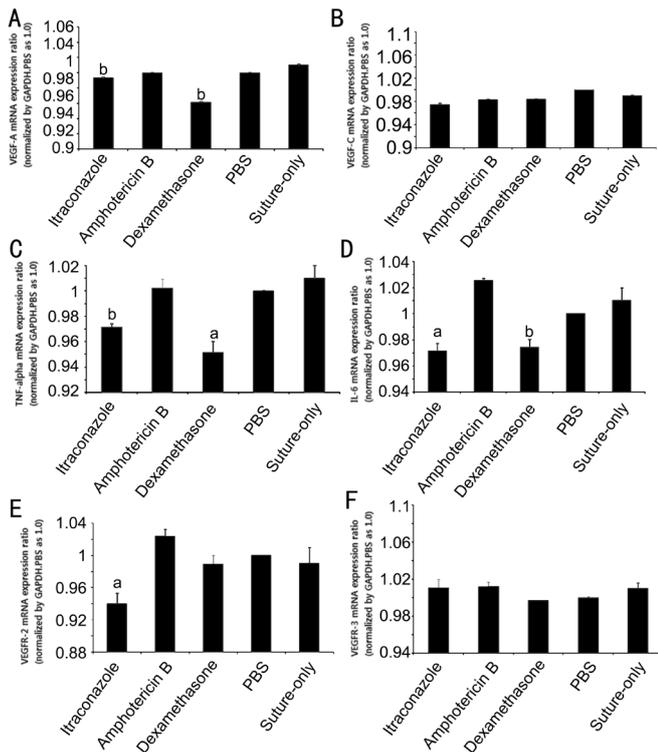
Figure 5 Comparison of mRNA expression among the five groups (itraconazole, amphotericin B, dexamethasone, PBS, suture-only groups) at 6wk post-sutures A: VEGF-A; B: VEGF-C; C: TNF-alpha; D: IL-6; E: VEGFR-2; F: VEGFR-3. mRNA expression ratios were normalized by GAPDH (PBS group=1.0). [a]$P<0.05$, [b]$P<0.01$ compared with PBS.

There was a significant difference in VEGFR-2 expression among the itraconazole, amphotericin B, and dexamethasone groups ($P<0.01$). The itraconazole group had a significantly lower VEGFR-2 level than the PBS and amphotericin B groups (ANOVA with post hoc test, $P=0.013$, <0.01, respectively) (Figure 5E).

There was no significant difference in VEGFR-3 expression among the three treatment groups ($P>0.05$) (Figure 5F).

Penetrating Keratoplasty Model

Graft survival Figure 6 shows the survival curve. Graft survival in the itraconazole group was higher than that in the amphotericin B, PBS groups and KP-only group (log-rank test, $P=0.057$, 0.096, 0.012, respectively). There was no difference in graft survival between the amphotericin B and PBS groups, nor between the dexamethasone and itraconazole groups, nor between the PBS and the KP-only group (all $P>0.05$). Graft survival in the dexamethasone group was higher than that in the PBS group ($P=0.0167$). The eight-week graft survival rate was 28.57% in the itraconazole group, 62.50% in the dexamethasone group, 12.50% in the PBS group, 0 in the amphotericin B group and 0 in the KP-only group. The mean survival time of each group was as follows: itraconazole (6.29±0.60wk), dexamethasone (6.75±0.58wk), amphotericin B (4.31±0.69wk), PBS (4.00±0.69wk) and KP-only group (3.50±0.57wk).
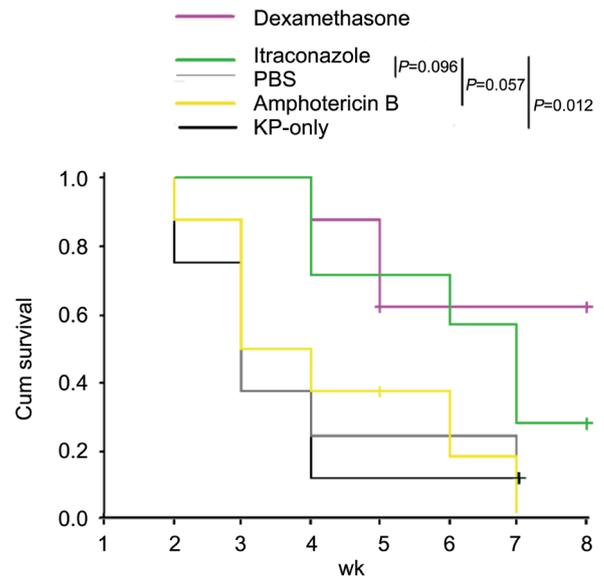

Figure 6 Comparison of graft survival among the five groups (itraconazole, amphotericin B, dexamethasone, PBS , KP-only groups).
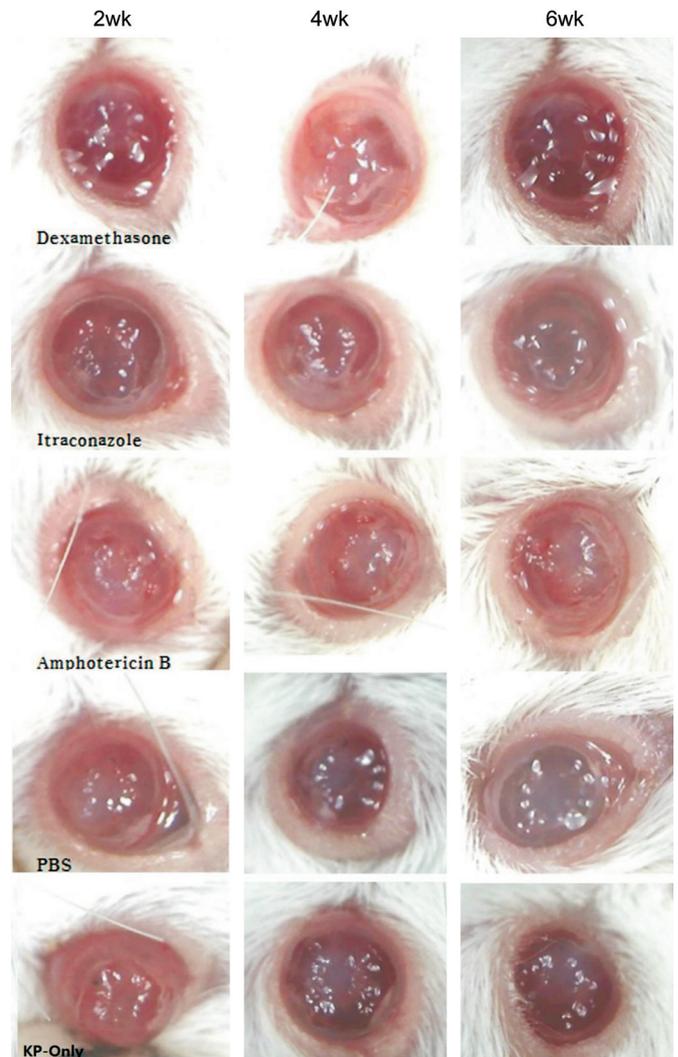

Figure 7 Biomicroscopic pictures of cornea after PK in five groups (itraconazole, amphotericin B, dexamethasone, PBS, KP-only groups).

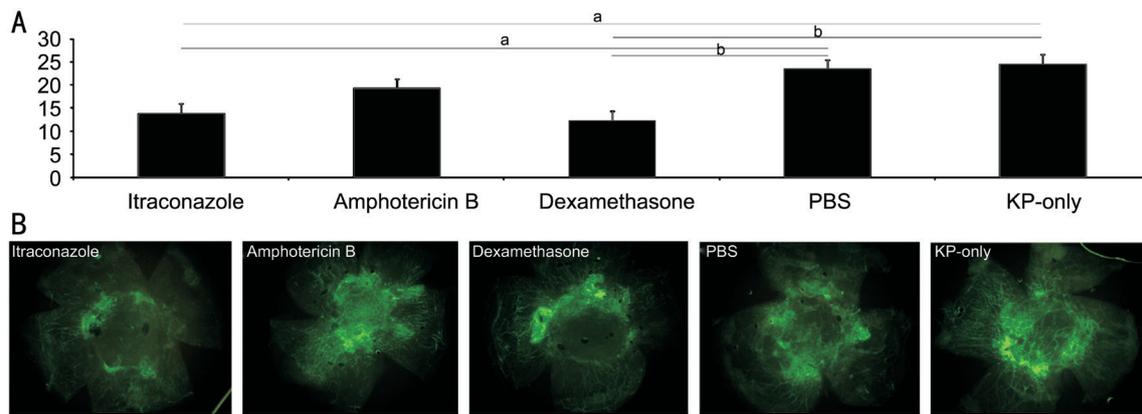Figure 7 shows biomicrocopic pictures of each five group after PK.

Figure 8 NV in PK model A: Comparison of NV in the PK model among the five groups (itraconazole, amphotericin B, dexamethasone, PBS, KP-only groups) at 8wk post-PK; B: Representative pictures. [a]$P<0.05$, [b]$P<0.01$.
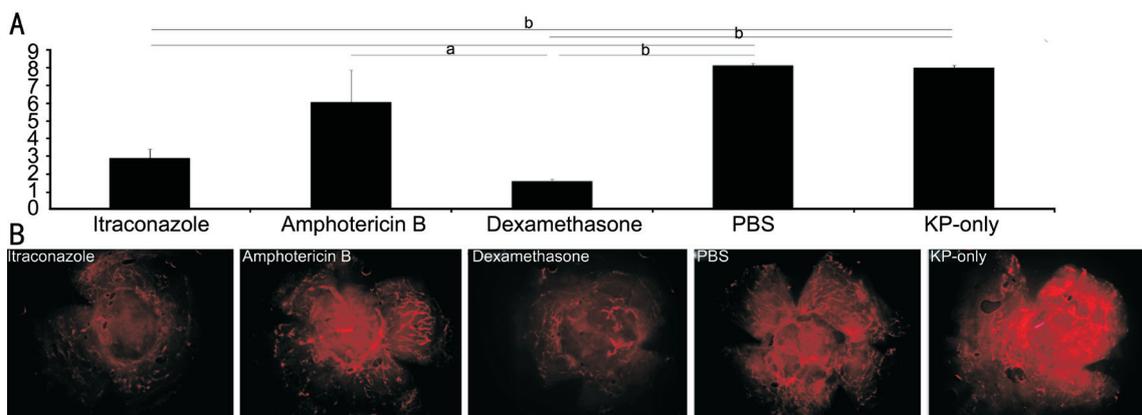

Figure 9 LY in PK model A: Comparison of LY in the PK model among the five groups (itraconazole, amphotericin B, dexamethasone, PBS, KP-only groups) at 8wk post-PK; B: Representative pictures. [a]$P<0.05$, [b]$P<0.01$.

Neovascularization The neovascularized area was smaller in the itraconazole (13.92%±2.14%) and dexamethasone (12.28%±2.02%) groups than in the PBS group (23.60%± 1.91%) (ANOVA with post hoc test, $P=0.024$, 0.008, respectively) (Figure 8). There was no difference in NV between the PBS and amphotericin B groups (19.35%±1.90%) nor between the PBS and KP-Only group (24.65%±2.01%).

Lymphangiogenesis There was less total LY in the itraconazole group (2.89%±0.51%) than the PBS group (8.14%±0.27%) ($P=0.001$) (Figure 9). There was less LY in the dexamethasone group (1.59%±0.16%) than the amphotericin B (6.05%±1.83%) and PBS groups (ANOVA with post hoc test, $P=0.011$, <0.001, respectively). There was no difference in LY between the PBS and the KP-only group (8.01%±0.13%).

DISCUSSION

Fungal keratitis is difficult to treat, especially in eyes that have undergone surgical intervention such as PK, for which steroids are used to prevent graft rejection[1]. Even after resolution of fungal keratitis, the clarity of a cornea with profuse NV, LY and opacity may not improve, resulting in decreased visual acuity. In this situation, antifungals that have anti-angiogenic and anti-inflammatory effects are essential.

Itraconazole and amphotericin B are both effective antifungals against various fungal infection[1-5,8,17-19] and both have previously been reported to have anti-angiogenic effect[7,18,20-21]. Amphotericin B binds with ergosterol, a component of fungal cell membranes, forming pores that cause rapid leakage of monovalent ions (K^+, Na^+, H^+ and Cl^-) and subsequent fungal cell death[1,18].

Itraconazole inhibits the fungal-mediated synthesis of ergosterol, a component of fungal cell membranes, *via* inhibition of lanosterol 14α-demethylase[1,17,20]. Lanosterol 14α-demethylase is also responsible for the synthesis of cholesterol in humans and essential for endothelial proliferation[7-8].

One suggested anti-angiogenic mechanism of itraconazole is the perturbation of cholesterol trafficking pathways by inhibiting lanosterol 14α-demethylase[7-8,22]. This would result in inhibition of mTOR activity and VEGFR-2 glycosylation in endothelial cells[6-7,23]. Itraconazole has been reported to inhibit Hedgehog signaling pathway, thereby suppressing the growth of tumors[6,23].

In contrast, the anti-angiogenic effect of itraconazole can be due to its direct stimulation of apoptosis in endothelial cells, not due to inhibition of VEGF signaling (independent of VEGF)[24].

Itraconazole has been shown to be an inhibitor of multiple angiogenic stimulatory pathways in previous studies[7-8,24]. In our study, NV in a corneal suture model and PK model was

significantly inhibited by itraconazole, and our RT-PCR results indicate that VEGF-A-VEGFR-2 is involved in this decrease in NV.

As well as anti-angiogenesis, itraconazole was reported to suppress LY by inhibiting tomor VEGF-C expression[9]. Our RT-PCR result showed that VEGF-C expression was less than PBS (P=0.098). VEGFR-3 expression did not decrease in response to itraconazole treatment, but itraconazole did decrease LY in the corneal suture model and PK model compared to the control group. We suspect that this LY may have been medicated by VEGF-C-VEGFR-2[15], or VEGF-A which induces proliferation of lymphatic endothelium as well as angiogenesis by inducing directly lymphatic endothelial cell proliferation through VEGFR-2, since this is strongly expressed on normal lymphatic endothelium[25-27].

Because it is difficult to clinically establish a diagnosis of fungal keratitis, the use of smears and cultures is of extreme importance. It is often difficult for a clinician faced with the possibility of a fungal keratitis to decide which antifungal to use.

In cases of *Candida spp.*, amphotericin B may be the drug of choice. Natamycin, voriconazole, itraconazole and fluconazole are also effective[1,4,19]. *Fusarium spp.*, keratitis treated successfully with natamycin, ketoconazole and amphotericin[1,4,19]. *Aspergillus spp.*, keratitis treated successfully with amphotericin, natamycin, voriconazole, itraconazole[1,4,19]. *Curvularia spp.*, keratitis treated successfully with amphotericin, clotrimazole, voriconazole, itraconazole[1,2,4,19].

With respect to antifungal spectrum, no single agent was universally most effective in fungal keratitis. Both amphotericin B and itraconazole are active against a broad spectrum of fungi. Amphotericin B is a preferred treatment if yeasts or pseudohyphae are seen in fungal keratitis[1,4,19]. However, topical amphotericin B is toxic and delays wound healing[2-5,28]. Topical administration of 1.5 to 5 mg/mL is commonly used administration route in the treatment of fungal keratitis[4].

Subconjunctival administration can be used but limited due to reports of conjunctival necrosis, scleritis, and scleral thinning[4]. In high dose intracorneal administration, it can cause endothelial cell loss and persistant corneal edema[4]. Itraconazole can be used effectively as topical and oral administration of infection by *Candida spp*[1,19,21]. Itraconazole is effective against *Aspergillus spp*, and other filamentous fungus[2,5], but ineffective in treatment of infection by *Fusarium spp*.

With respect to anti-inflammatory effect, amphotericin B did not have an anti-inflammatory effect, whereas itraconazole had a significant anti-inflammatory effect in our study. Even though amphotericin B is the first line treatment when yeasts and pseudohyphae are seen, itraconazole can be effectively applied in the clinic rather than amphotericin B considering the lower toxicity and the high anti-inflammatory effect.

In our PK model, graft survival was better in the itraconazole group than the amphotericin B and PBS groups (P=0.096,

0.057, respectively). There was no difference in angiogenesis, LY, or inflammatory infiltration between the amphotericin B group and PBS group in either the PK or suture model despite the previously reported[18] anti-angiogenic effect of amphotericin B.

Dexamethasone is known to be an effective anti-angiogenic agent; consistent with this, the dexamethasone group showed significantly less NV, less LY, less inflammation, and greater graft survival than the PBS and amphotericin B group (all P<0.05). There was no statistically significant difference between the dexamethasone and itraconazole groups in graft survival in the PK model (P=0.36) and no statistically significant difference in NV, LY, or inflammation in the suture model.

This suggests that itraconazole has comparable anti-angiogenic and anti-inflammatory effect to those of dexamethasone. In cases of fungal keratitis, however, corticosteroids should be approached cautiously, because fungal growth in tissue is aided by corticosteroids. Though, the use of topical corticosteroids in the postoperative management of fungal keratitis is controversial, topical corticosteroids can be tried cautiously to decrease corneal inflammation and scarring[29-32]. At the time of keratoplasty, if the infection has been controlled clinically with total, wide removal of the lesion, topical corticosteroids can be used. Generally, after corneal transplantation in fungal keratitis, we can use topical steroids cautiously after 2 to 4wk after surgery.

Itraconazole, in addition to its intrinsic broad anti-fungal activity, has anti-(lymph)angiogenic and anti-inflammatory effects, which can be safely used to decrease corneal inflammation and angiogenesis in fungal keratitis.

Thus, itraconazole can be one of therapeutic option in corneas with fungal keratitis, especially in corneas with surgical intervention in which we essentially need to choose less inflammatory and less angiogenic antifungals.

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