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Comparison of corneal collagen cross-linking (PACK-CXL) and voriconazole treatments in experimental fungal keratitis

Hüseyin Baran Özdemir,[1] Ayşe Kalkancı,[2] Kamil Bilgihan,[3] Pınar Uyar Göçün,[4] Betül Öğüt,[4] Funda Karakurt[2] and Merve Erdoğan[2]

[1]Ulucanlar Eye Training and Research Hospital, University of Health Sciences, Ankara, Turkey
[2]Department of Medical Microbiology, Gazi University Faculty of Medicine, Ankara, Turkey
[3]Department of Ophthalmology, Gazi University Faculty of Medicine, Ankara, Turkey
[4]Department of Pathology, Gazi University Faculty of Medicine, Ankara, Turkey

ABSTRACT.

Purpose: **To compare the antifungal efficacy of corneal collagen cross-linking with photoactivated riboflavin (PACK-CXL) and voriconazole in experimental *Fusarium solani* and *Candida albicans* keratitis models.**

Methods: **Sixty-four corneas of 32 New Zealand rabbits were included and divided into two main groups. Intrastromal injection of *Fusarium* and *Candida* suspensions was performed, and it was observed that keratitis was formed on the third day. Both groups were randomly separated into the following four groups: control, PACK-CXL, voriconazole and PACK-CXL combined with voriconazole. PACK-CXL was applied using 0.25% riboflavin in an accelerated Dresden protocol (total ultraviolet A dose 5.4 J/cm²). Voriconazole was applied topically as 7x1/day with a dose of 1% (10 mg/ml). Corneal buttons were excised on the tenth day, and microbiological and pathological examinations were performed.**

Results: **The PACK-CXL and PACK-CXL combined with voriconazole groups each had 100 colony-forming unit (CFU/ml) of reproduced micro-organisms compared with 500 CFU/ml in the voriconazole group and 1500 CFU/ml in the control group ($p < 0.001$) in the *Fusarium* keratitis model. The PACK-CXL combined with voriconazole group had 100 CFU/ml, the PACK-CXL group had 150 CFU/ml, and the voriconazole group had 200 CFU/ml of reproduced micro-organisms compared with 4000 CFU/ml in the control group ($p < 0.002$) in the *Candida* keratitis model. ($p < 0.001$). Fewer hyphae and non-specific stromal changes were observed in the pathological cross sections examined in subgroups that used CXL.**

Conclusion: **There was less fungus reproduction and a lower keratitis score for *Fusarium solani* and *Candida albicans* in the treatment groups compared to the control groups, especially in groups that used PACK-CXL. These results suggest that it is useful to combine PACK-CXL treatment with medical treatment in the fungal keratitis algorithm at the early stage of the disease.**

Key words: corneal cross-linking – fungal keratitis – PACK-CXL – voriconazole

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Introduction

Fungal keratitis constitutes an important cause of blindness in both developed and developing countries (Ou & Acharya 2007). Clinical studies show that *Fusarium* and *Aspergillus* (both moulds) and *Candida* (a yeast) are the most common organisms that cause fungal keratitis (Kalkanci & Ozdek 2011). Although the treatment of fungal keratitis is difficult and expensive, there are cases in which corneal perforation or endophthalmitis cannot be prevented despite having optimal treatment provided (Richoz et al. 2014). Medical treatment is mostly chosen empirically. Surgical treatment is applied to patients who already have or are about to have corneal perforation.

The positive effect of riboflavin and ultraviolet A (UVA) combination on corneal infections was shown in 2008, for the first time, by Iseli et al. (2008) in five patients with keratitis resistant to conservative treatment. Retrospective and prospective studies on human keratitis showed that corneal collagen cross-linking (CXL) may have an important role in microbial keratitis treatment (Vazirani & Vaddavalli 2013; Khater et al. 2014; Said et al. 2014; Shetty et al. 2014). Later, there were many publications showing that CXL may be effective in keratitis treatment based on many case reports (Alio et al.

2013). In the Alio et al. article, 'Photoactivated Chromophore for Infectious Keratitis', corneal collagen cross-linking (PACK-CXL) terminology was first suggested, and the terminology was later accepted in the Ninth Annual International Corneal Cross-linking Congress in Dublin in 2013 (Hafezi & Randleman 2014).

The number of randomized controlled *in vitro* and *in vivo* studies is limited for PACK-CXL. Although PACK-CXL was shown in studies to be effective against bacterial factors *in vitro* and *in vivo*, there were conflicting results when PACK-CXL was used against fungal factors (Martins et al. 2008; Makdoumi et al. 2010; Sauer et al. 2010; Galperin et al. 2012). There are also publications detailing that PACK-CXL treatment applied to fungal strains *in vitro* does not have a satisfactory fungicidal effect (Martins et al. 2008). This lack of efficacy may be explained by the unsuitability of parameters in PACK-CXL treatment for fungal factors. Additionally, in the *in vitro* study made by Bilgihan et al., 0.1% and 0.25% concentrations of riboflavin were used during PACK-CXL treatment and showed that the PACK-CXL treatment combined with a 0.25% riboflavin concentration had a higher fungicidal rate (Bilgihan et al. 2016). No additional previous experimental animal studies comparing the effect of PACK-CXL treatment and antifungal treatment in fungal keratitis are available.

The aim of our study was to investigate the efficiency of PACK-CXL treatment with 0.25% riboflavin concentration and to compare with topical voriconazole treatment in *Fusarium solani* and *Candida albicans* keratitis models experimentally formed in rabbit eyes based on clinical, microbiological and pathological analyses.

Materials and Methods

This study was approved by the Animal Experiments Local Ethical Board (G.Ü.E.T-15.039) at the University of Gazi (Ankara, Turkey). The study was conducted in Gazi University Laboratory Animals Breeding and Experimental Researches Centre and Eye Diseases Department.

Thirty-two adult New Zealand rabbits each weighing 2.5–3 kg were used in the study. Both eyes of all rabbits were included in the study. Operations were made under general anaesthesia using intramuscular ketamine and xylazine. Topical proparacaine HCl 0.5% (Alcaine; Alcon Pharmaceuticals Ltd., Puurs, Belgium) was applied before every operation. The rabbits were divided into two groups (group 1 for *Fusarium solani* and group 2 for *Candida albicans*). Fungal suspensions with a concentration of 10^5 colony-forming unit (CFU/ml) (from *Fusarium solani* and *Candida albicans* strains prepared by Gazi University Microbiology Department and acquired from human keratitis patients in their clinic) were injected intrastromally into the cornea in both eyes of the rabbits with a dose of 0.1 ml. In the examination on the third day, the formation of keratitis was observed in all rabbit corneas. Fungal agents were isolated from corneal scraping samples of clinically suspected keratitis cases. Both *F. solani* and *C. albicans* were identified morphologically. Colony morphology and conidia morphology were used for *F. solani* identification. Germ tube test positivity and carbohydrate assimilation profiles were used for *C. albicans* identification.

Both keratitis models (group 1 for *Fusarium* keratitis model and group 2 for *Candida* keratitis model) were divided into four subgroups, each group comprising eight eyes. The first subgroups (groups 1A and 2A) were selected as control groups, and no treatment was given. The other subgroups were the PACK-CXL (groups 1B and 2B), voriconazole (groups 1C and 2C) and PACK-CXL combined with voriconazole (groups 1D and 2D) groups. PACK-CXL treatment was performed on the third day for groups 1B, 1D, 2B and 2D. Topical treatment with 1% (10 mg/ml) voriconazole drops 7x1/day between the third and tenth days was applied to the eyes in groups 1C, 1D, 2C and 2D.

Riboflavin at a concentration of 0.25% was used for PACK-CXL. By applying riboflavin at a concentration of 0.25% once every 5 min for 30 min, penetration of riboflavin into the cornea was provided. Ultraviolet A (UVA) light of 365 nm wavelength (CCL-Vario; Peschke Meditrade GmbH, Hünenberg, Switzerland) was calibrated at 9 mW/cm^2 and applied to the cornea from a distance of 10 mm for 10 min. The total dose was 5.4 J/cm^2 with this irradiation procedure.

On the third day (before PACK-CXL treatment) and tenth day (on the seventh day after PACK-CXL treatment) after intrastromal fungal strain injection, the biomicroscopic examination was made in all rabbit eyes after the anaesthesia described above. Intrastromal injection, third-day and tenth-day examination findings of all eyes were recorded with images taken using a microscope camera. Keratitis degree was evaluated by modifying the scale developed by Schreiber et al. (2003). Corneal clouding, corneal neovascularization (NV), conjunctival hyperaemia, corneal infiltrate and hypopyon degree were evaluated with this scale. Corneal haze was scored as follows: no oedema, 0; minor oedema, 1; corneal oedema in two quadrants, 2; and total corneal oedema, 3. Corneal NV was graded as 0, clear cornea; 1, NV up to 2 mm from the limbus; 2, NV more than 2 mm from the limbus; and 3, NV that advanced to the centre of the cornea. Conjunctival hyperaemia was graded as follows: no hyperaemia, 0; light hyperaemia, 1; moderate hyperaemia, 2; and severe hyperaemia, 3. Hypopyon level and the size of corneal infiltrate diameter were measured in millimetres. Using these criteria, keratitis scores were calculated by one examiner (HBO).

At the end of the study, in each subgroup, corneal buttons were excised from the limbus, and seven corneal buttons were given to Gazi University Microbiology Department for culture cultivation. The corneal buttons were culture cultivated under sterile conditions. Cornea samples were cultivated on Sabouraud Dextrose Agar, blood agar and Eosin Methylen-blue Lactose Sucrose agar plates, and the reproduced colonies were followed up in the microbiology laboratory. The number of colonies was recorded. The reproduced colonies were redefined and named as *Candida albicans* and *Fusarium solani*.

One of the corneal buttons in each subgroup was given to Gazi University Pathology Department for pathological examination. Four-μm-thick cross sections of tissues (detected with 10% formaldehyde) were taken on positively charged slides. Staining was made in a Ventana Benchmark Special Stain BSS device (F. Hoffmann-La Roche AG, Basel, Switzerland) using PAS, Giemsa and haematoxylin and eosin instant staining kits. The examination was performed by two pathologists (PUG,

BO), and three main parameters were evaluated: inflammation, stromal oedema and yeast/hyphae density. Inflammation was graded as follows: density of inflammatory cells less than 25% of corneal thickness, low; density of inflammatory cells between 25% and 50% of corneal thickness, moderate; and density of inflammatory cells more than 50% of corneal thickness, intense. Stromal oedema was graded as follows: oedema less than 25% of corneal thickness, low; oedema between 25% and 50% of corneal thickness, moderate; and oedema more than 50% of corneal thickness, intense. Yeast/hyphae density was graded as follows: no yeast/hyphae seen, none; yeast/hyphae seen but no mass formation seen, low; yeast/hyphae mass formation seen but less than 25% of corneal thickness, moderate; and yeast/hyphae mass formation seen but more than 25% of corneal thickness, intense.

The data were analysed with IBM SPSS Statistics 17.0 (IBM Corporation, Armonk, NY, USA). Descriptive statistics were shown as the median (minimum - maximum) for constant numerical and gradable variables. The significance of the difference of median values among the subgroups was examined with Mann–Whitney *U* test when there were two independent subgroups and with Kruskal–Wallis test for more than two independent subgroups. Wilcoxon signed rank test was used to examine whether there was a statistically significant difference between Schreiber scores of the third and tenth days among the subgroups. Unless stated otherwise, the results at $p < 0.05$ were accepted as statistically significant. However, Bonferroni correction was made to be able to control for Type I errors in all possible multiple comparisons.

Results

Biomicroscopic findings

No statistically significant difference was observed in the average modified Schreiber scores between the third day and tenth day among subgroups in both keratitis trials according to Bonferroni correction ($p > 0.00625$) (Table 1). The average modified Schreiber score was lower in groups 1B, 1C and 1D in comparison with group 1A and was statistically significant ($p < 0.006$) (Table 2). The average modified Schreiber score was significantly lower in group 2B, group 2C and group 3D when compared to group 2A ($p < 0.002$).

Microbiological findings

There was a statistically significant difference in median CFU counts among subgroups in the *Fusarium* keratitis model ($p < 0.001$), and the median corneal CFU count decreased in groups 1B, 1C and 1D compared to group 1A ($p < 0.001$) Fig. 1. Lower CFU counts were found in group 1B and group 1D compared to group 1C ($p < 0.001$) (Table 3).

There was a statistically significant difference in median CFU counts

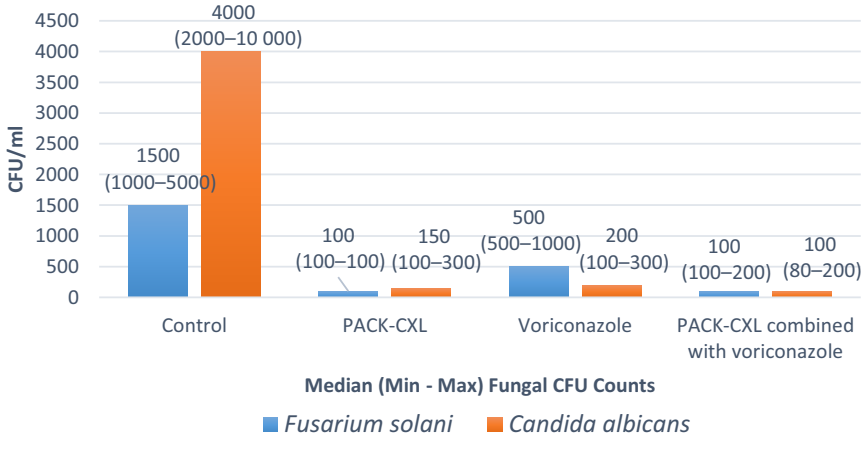


Fig. 1. Bar graph of cell counts (CFU/ml) of *Fusarium solani* and *Candida albicans*. *Y*-axis demonstrates median of cell counts (log CFU/ml). *X*-axis demonstrates four different groups of study in both keratitis models.

Table 2. Tenth-day modified Scheiber scores according to the groups.

	Fusarium solani	*Candida albicans*
Control	2.6 (1.5–3.5)	3.8 (2.9–4.9)
PACK-CXL	1.7 (1.1–2.5)	2.0 (0.8–2.4)
Voriconazole	1.4 (0.9–2.2)	1.9 (1.5–2.3)
PACK-CXL combined with voriconazole	1.2 (0.9–1.9)	1.7 (1.2–2.8)
p-value	0.006	0.002

Descriptive statistics are shown as the median (minimum - maximum). The test used for statistical analysis among groups in both *Fusarium solani* and *Candida albicans* keratitis models was Kruskal–Wallis test with a Bonferroni correction, and the results with $p < 0.0125$ were accepted as statistically significant. The modified Schreiber score was significantly lower in the treatment groups compared to the control group in both keratitis models.

Table 1. Modified Scheiber scores on the third and tenth days among subgroups.

	Fusarium solani			*Candida albicans*		
	Third day	Tenth day	p-value	Third day	Tenth day	p-value
Control	1.3 (1.0–1.8)	2.6 (1.5–3.5)	0.018	2.8 (2.1–3.7)	3.8 (2.9–4.9)	0.018
PACK-CXL	2.1 (0.9–2.9)	1,7 (1.1–2.5)	0.043	2.9 (1.9–3.5)	2.0 (0.8–2.4)	0.018
Voriconazole	1.5 (0.6–2.1)	1.4 (0.9–2.2)	0.866	2.6 (2.2–2.8)	1.9 (1.5–2.3)	0.018
PACK-CXL combined with voriconazole	1.5 (0.8–2.9)	1.2 (0.9–1.9)	0.028	2.9 (2.2–3.6)	1.7 (1.2–2.8)	0.018

Descriptive statistics are shown as the median (minimum–maximum). For the comparisons made between the third and tenth days among the groups, the results with $p < 0.00625$ were accepted as statistically significant according to Wilcoxon signed rank test and Bonferroni correction. There was no statistically significant difference in the modified Schreiber scores between the third day and tenth day among groups in both keratitis models.

Table 3. Colony-forming unit (CFU) counts (CFU/ml) of micro-organisms according to the groups.

	Fusarium solani	*Candida albicans*
Control	1500 (1000–5000)	4000 (2000–10 000)
PACK-CXL	100 (100–100)	150 (100–300)
Voriconazole	500 (500–1000)	200 (100–300)
PACK-CXL combined voriconazole	100 (100–200)	100 (80–200)
p-value	<0.001	0.002

Descriptive statistics are shown as the median (minimum - maximum). For the comparisons made between *Fusarium solani* and *Candida albicans* among the groups, the results with $p < 0.0125$ were accepted as statistically significant according to Mann–Whitney *U* test and Bonferroni correction. CFU counts were significantly lower in the treatment groups, especially in groups with PACK-CXL, compared to the control groups in both keratitis models.

Table 4. Pathological examination results.

Groups	Inflammation	Stromal oedema	Yeast/Hyphae
Group 1A	Moderate	Moderate	Low
Group 1B	Moderate	Low	None
Group 1C	Moderate	Low	Low
Group 1D	Moderate	Low	None
Group 2A	Intense	Moderate	Intense
Group 2B	Intense	Moderate	None
Group 2C	Intense	Moderate	Moderate
Group 2D	Low	None	None

Inflammation was graded as follows: density of inflammatory cells less than 25% of corneal thickness, low; density of inflammatory cells between 25% and 50% of corneal thickness, moderate; and density of inflammatory cells more than 50% of corneal thickness, intense. Stromal oedema was graded as follows: oedema less than 25% of corneal thickness, low; oedema between 25% and 50% of corneal thickness, moderate; and oedema more than 50% of corneal thickness, intense. Yeast/hyphae density was graded as follows: no yeast/hyphae seen, none; yeast/hyphae seen but no mass formation seen, low; yeast/hyphae mass formation seen but less than 25% of corneal thickness, moderate; and yeast/hyphae mass formation seen but more than 25% of corneal thickness, intense.

among subgroups in the *Candida* keratitis model ($p = 0.002$), and the median corneal CFU count decreased in groups 2B, 2C and 2D compared to group 2A ($p < 0.001$) (Table 3). Lower CFU counts were found in group 2B and group 2D compared to group 2C ($p = 0.009$ and $p = 0.002$).

Pathological findings

Pathological examination results are shown in Table 4 (Figs 2 and 3). When these results were compared with the control group, it was observed that inflammation and stromal oedema had decreased mildly in all subgroups. It was also observed that the yeast-and-hyphae decrease ratio was higher in PACK-CXL applied subgroups (groups 1B, 1D, 2B and 2C) compared to the groups in which only voriconazole treatment was applied.

Discussion

Fungal keratitis is an important cause of morbidity due to the difficulty of treatment, the frequency of late diagnoses and progression despite treatment. *Fusarium* and *Aspergillus* are the moulds, and *Candida* are the yeasts most commonly causing fungal keratitis (Kalkanci & Ozdek 2011). Thus, in our study, experimental keratitis was formed with *Fusarium solani* and *Candida albicans* fungi and treatment groups were compared. Voriconazole was selected for use in our study due to its high corneal penetration when administered topically and its success in fungal keratitis treatment in recent years (Hariprasad et al. 2008). PACK-CXL combined with a 0.25% riboflavin concentration was found to have high fungicidal effect in 10^4 CFU/ml and against lower fungal concentrations, and it was observed that the fungicidal effect of PACK-CXL with a 0.1% riboflavin concentration had a lower fungicidal effect (Bilgihan et al. 2016). For this reason, solutions containing a 0.25% riboflavin concentration were used in our study when applying PACK-CXL, and a total of 5.4 J/cm^2 UVA was applied as standard.

In our study, we found that PACK-CXL is as effective as voriconazole in decreasing fungal CFU counts in *Fusarium solani* and *Candida albicans* keratitis models. Additionally, PACK-CXL has a synergistic effect with voriconazole on CFU count, exhibiting a decrease when compared to groups treated with only PACK-CXL. In addition, microscopic examination findings and pathological results showed that clinical features do not improve as fungal counts decrease.

The number of published *in vivo* animal studies investigating the effect of PACK-CXL is low. Cosar et al. (2015) reported that the efficacy of PACK-CXL was not satisfactory in *Pseudomonas* keratitis by itself but had a synergistic effect with topical moxifloxacin treatment. Tal et al. clinically compared PACK-CXL treatment and the amount of decrease in ulcer dimensions. The shortest recovery duration was observed in the PACK-CXL group in this study (Tal et al. 2015). The only *in vivo* animal study investigating the effect of PACK-CXL on fungal keratitis was made by Galperin et al. (2012) in experimental *F. solani* keratitis formed in rabbits. The control group was compared with a PACK-CXL group in this study, and the culture reproduction and clinical scores were lower in the PACK-CXL group.

The PACK-CXL treatment, when used alone or combined with voriconazole, seems to be effective in *F. solani* and *C. albicans* keratitis models. The low decrease in inflammation causes us to surmise that, although yeast and hyphae formations decrease in the early period, the regression of inflammation takes longer. Riboflavin solution at a 0.1% concentration was used with PACK-CXL in other studies, and 0.25% was used in our study. Increased fungicidal rates in our study made us consider that the increased riboflavin concentration increased the efficiency of PACK-CXL, as supported by previous *in vitro* studies.

When large case clinical studies and case series on PACK-CXL are

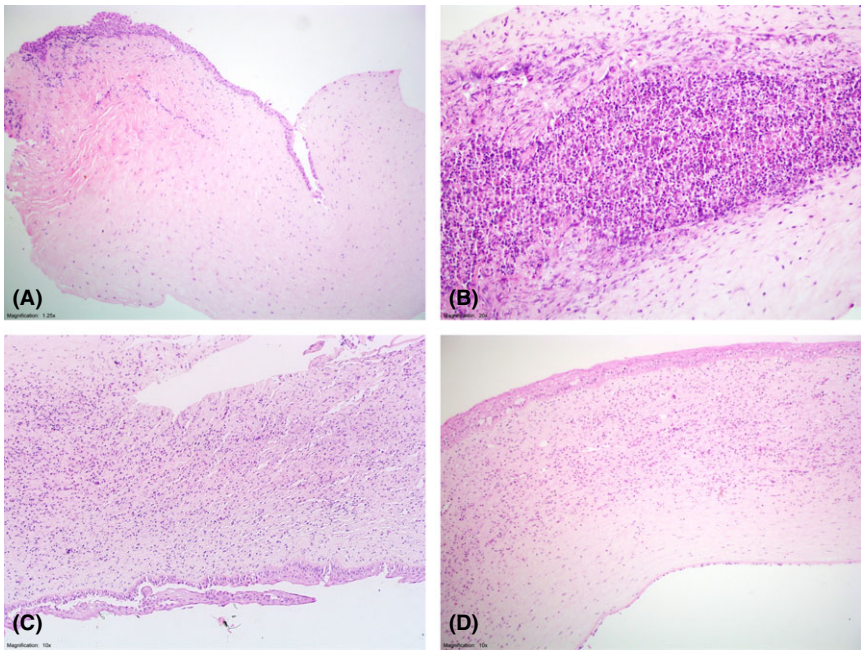


Fig. 2. Pathological cross sections of the experimental group in which *Fusarium solani* keratitis was formed are available in the figure. (A) Group 1A for the control group of *F. solani* – H&E × 40, (B) Group 1B for the PACK-CXL group of *F. solani* – H&E × 200, (C) Group 1C for the voriconazole group of *F. solani* – H&E × 100, (D) Group 1D for the PACK-CXL combined with voriconazole group of *F. solani* – H&E × 40.

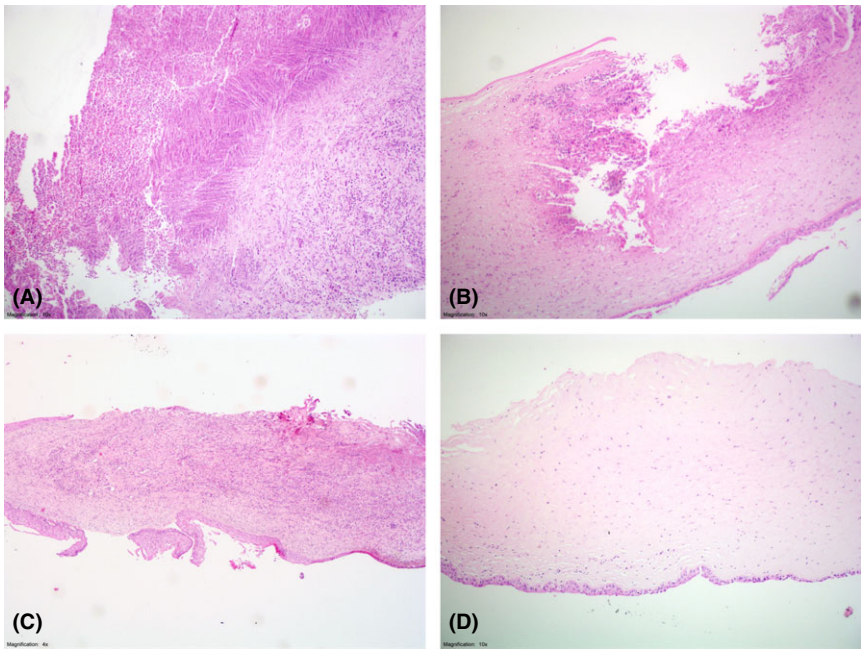


Fig. 3. Pathological cross sections of the experimental group in which *Candida albicans* keratitis was formed are available in the figure. (A) Group 2A for the control group of *C. albicans* – H&E × 100, (B) Group 2B for the PACK-CXL group of *C. albicans* – H&E × 100, (C) Group 2C for the voriconazole group of *C. albicans* – H&E, ×40, (D) Group 2D for the PACK-CXL combined with voriconazole group of *C. albicans* – H&E × 40.

examined, it is observed that the efficiency of this treatment is higher in bacterial keratitis and that keratitis complications occur more often in fungal keratitis (Price et al. 2012; Said et al. 2014; Shetty et al. 2014; Uddaraju et al. 2015). It is thought that the effect of PACK-CXL decreases with deep stromal ulceration and late treatment. We started the treatment on the third day, and considering previous clinical and laboratory studies, our study confirms that the efficiency of PACK-CXL treatment is higher in the early period when ulcerative keratitis diagnosis is certain.

This study made us consider that PACK-CXL may provide satisfactory antifungal effect despite the opinions stating that PACK-CXL is less successful in fungal keratitis. The inclusion of PACK-CXL treatment in the fungal keratitis treatment algorithm, especially in combination with antifungal drugs and applied at an early phase, was considered to be appropriate. Additionally, increasing the riboflavin concentration in PACK-CXL may enhance the antimicrobial effect. This study may lead the way for additional prospective randomized studies with strengthened PACK-CXL.

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Correspondence:
Hüseyin Baran Özdemir, MD
Ulucanlar Goz Egitim ve Arastirma Hastanesi
Kale Mh. Ulucanlar Cd. No:59, 06250,
Altındağ
Ankara
Turkey
Tel: +90 505 871 92 39
Fax: +90 312 312 38 05
Email: baranozdemir@gmail.com

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