Comparison of intravitreal antifungal 100 µg voriconazole and 5 µg amphotericin B in experimental *Aspergillus flavus* endophthalmitis model in rabbits

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ABSTRACT

BACKGROUND Fungal endophthalmitis is a sight-threatening disease associated with high morbidity and *Aspergillus sp*. is the most common causes. Voriconazole (VCZ) and Amphotericin B (AmB) are the most used antifungal drugs, while head-to-head comparison for *in vivo* intravitreal efficacy is still unknown. This study was aimed to compare the efficacy of both agents against *Aspergillus flavus*.

METHODS A randomized, masked, controlled-experimental study was conducted on 15 albino New Zealand white rabbits. Endophthalmitis was induced by intravitreal inoculation of *Aspergillus flavus*. Intravitreal injection was given 24 hours post-inoculation, the rabbits were divided into three groups; 100 µg/0.1 ml VCZ intravitreal injection, 5 µg/0.1 ml AmB, and control. Clinical evaluation of corneal opacity, aqueous cells and flare, and vitreous opacity using Yang's method of quantification were performed at day 1, 3, 5, 7, and 10 after treatment. Mycology quantitative analysis and histopathological examination were performed at the final evaluation.

RESULTS Clinical evaluation showed improvement of inflammation in the VCZ and AmB treatment groups (Δ score –2.1 [2.8] and –1.0 [3.2]) compared with the control group (Δ score 0.8 [3.1]). Although the VCZ group demonstrated a better clinical response with less inflammation and relatively intact retina structures in the histopathology result. Number of fungal colony was significantly less in AmB group (CFU/0.1 ml, $p < 0.05$).

CONCLUSIONS Favorable clinical improvement was shown in VCZ group compared to AmB group. Intravitreal VCZ showed a better clinical response tendency for *Aspergillus flavus*-induced endophthalmitis in rabbits.

KEYWORDS amphotericin B, *Aspergillus flavus*, endophthalmitis, voriconazole

Fungal endophthalmitis (FE) is a sight-threatening infection of the intraocular fluids and tissue. It is classified as an emergency in ophthalmology and remained as one of the most important causes of visual morbidity with poor outcome, regardless of maximum treatment.[1,2] Despite being rare and covering only a small percentage of all exogenous endophthalmitis cases in developed countries (4.6–16.7% incidence for postoperative endophthalmitis), FE has a higher incidence in developing countries with a tropical climate. Fungi have been reported in India as the etiological cause of postoperative endophthalmitis in 21.8% of cases.[3] Our center reported a 4% incidence rate of postoperative FE from 2011 to 2013, with *Aspergillus flavus* as the sole etiological

cause.[5] *Aspergillus* sp. has been reported as the most commonly isolated agent in postoperative FE, comprising up to 38–74% of cases, with a more fulminant and destructive nature compared with other fungi, often resulting in evisceration.[5–7] *A. flavus* has been reported to have a 100-fold virulence compared with other *Aspergillus* sp.[8]

The variety of antifungal choice available is limited, in contrast to the wide array of fungal pathogens, creating a challenge in the management of FE.[9] Currently, amphotericin B (AmB) is the most common intravitreal antifungal agent used worldwide. It works by altering the cell membrane permeability through ergosterol binding, which ultimately leads to cell death.[10] However, it has a broad coverage against various fungi, although intravitreal use is also associated with retinal toxicity.[9] Voriconazole (VCZ) is a second-generation triazole, derived from synthetic fluconazole, with a broader spectrum of antifungal activity against common ocular pathogen and less retinal toxicity.[9,11] It exhibits antifungal activity through inhibition of cytochrome P-450 mediated 14-α demethylation enzyme.[9,12] The effectiveness of VCZ for FE has been demonstrated.[13] Both agents are currently used extensively for the treatment of FE; however, the *in vivo* comparison of efficacy between intravitreal VCZ and AmB is yet unknown. Therefore, this study was aimed to demonstrate the efficacy of intravitreal VCZ compared with that of AmB for the treatment of exogenous *A. flavus* endophthalmitis in a rabbit model.

METHODS

This study was a randomized, masked, controlled-experimental study using a rabbit model, conducted from July to August 2015 at the Health Research and Development Institution Animal Laboratory, Jakarta. Ethical approval was obtained from the Ethics Committee of Faculty of Medicine, Universitas Indonesia (No: 431/UN2.F1/ETIK/2015).

In vitro study

Susceptibility testing for VCZ and AmB was performed using disc diffusion method, with the CLSI M44-A document as the guideline.[14] VCZ (1 µg), AmB (20 µg) discs, Mueller-Hinton agar supplemented with 2% glucose, and methylene blue (0.5 mg/l) were used, incubated at 35°C, and evaluated at 24 hours. Zone diameters were read using the clear zone marker where growth decreased sharply.

Preparation of intravitreal antifungal agents

AmB deoxycholate (Amphot®, Lyka Lab) was reconstituted with sterile water to reach a concentration of 5 µg/0.1 ml. VCZ (VFend®, Pfizer) was reconstituted with 0.9% NaCl to a concentration of 100 µg/0.1 ml. All preparations were performed in a sterile condition to avoid contamination, in an individual syringe, masked from the researcher.

Animal model of exogenous *A. flavus* endophthalmitis

The right eyes of 15 New Zealand albino rabbits were used in this study. The animals were obtained from the Animal Research Institution, Ciawi, Indonesia. The animals weighed between 2.5 and 3.5 kg and aged around 4 months. All animals were individually housed in a controlled environment, with no restriction of food and water, and treated according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animal in Ophthalmic and Vision Research.[15] Each rabbit had been declared healthy and free of ocular abnormalities.

All rabbits were anesthetized before surgical procedure using an intramuscular injection of ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg). Pupils were dilated using 2.5% phenylephrine hydrochloride (Cendo Efrisel, Indonesia) and 1% tropicamide (Cendo Mydriatil, Indonesia). Topical anesthesia was given using 0.5% tetracaine hydrochloride (Cendo Pantocain, Indonesia).

The *A. flavus* isolate used in this study was obtained from the culture collection of the Mycology Division of Department of Parasitology, Faculty of Medicine, Universitas Indonesia. The isolate was grown on Sabouraud dextrose agar (SDA) for 72 hours at 25–29°C and was previously proven to be susceptible to both VCZ and AmB using disc diffusion method. *A. flavus* suspension was prepared by serial dilution to achieve the concentration equal to 0.5 McFarland standard. The rabbits were examined using handheld slit-lamp (Kowa SL-15) and indirect ophthalmoscope (Neitz IO-α) before injection. Anterior chamber paracentesis of 0.1 ml aqueous fluid was performed using a 30 gauge needle, and 0.1 ml of fungal suspension was introduced into

the vitreous cavity via *pars plana*, 2 mm posterior to the superotemporal limbus, with the bevel of the needle facing anteriorly to avoid puncturing the lens. Intravitreal injection was performed slowly, under loupe magnification, by a masked researcher under the supervision of a veterinarian.

Treatment groups

All 15 rabbits showed clinical signs of endophthalmitis 24 hours after *A. flavus* inoculation and were randomly distributed into two treatment groups and one control group. Six eyes were treated with 100 µg/0.1 ml VCZ (Group I) and another six with 5 µg/0.1 ml AmB (Group II), whereas three eyes received no treatment as control (Group III). VCZ was given intravitreally 24 hours after fungal inoculation, and the same injection was repeated 48 and 72 hours later, giving a total of three injections. AmB was given intravitreally 24 hours after fungal inoculation as a single injection.

Clinical evaluation

The intraocular inflammatory reactions of the anterior chamber and vitreous were graded using a method similar to Yang et al.[16] The severity of inflammation was evaluated by two masked observers on days 1, 3, 5, 7, and 10 post-treatment using handheld slit-lamp and indirect ophthalmoscope.

Mycological examination

On day 10 after treatment, approximately 0.3 ml of vitreous fluid was aspirated from all eyes. Direct smear examination using 10% potassium hydroxide (KOH) and SDA culture was performed. Samples were incubated for 72 hours at 25–29°C. All positive growth was identified morphologically using lactophenol cotton blue dye to verify the growth of *A. flavus*, recultured, and tested for its susceptibility against VCZ and AmB using disc diffusion method. The culture growth was also quantified as colony-forming unit (CFU) per milliliter.

Histopathological examination

The rabbits were euthanatized with intravenous 50 mg/kg sodium pentobarbital on day 10, afterward, the eyes were enucleated and placed in 10% buffered formalin as fixation solution for at least 24 hours. The eyes were then divided into two equal parts and embedded in paraffin. The sections were cut in 5 µm depth and stained with hematoxylin and eosin (H&E). Periodic acid-Schiff (PAS) staining was performed on one random sample from each group to visualize the fungal structure. Intraocular inflammatory changes were graded with a light microscope, using a histopathological grading scale similar to Lee et al.[17]

Statistical analysis

Data were analyzed using SPSS software, version 20 (IBM). Statistical significance between the VCZ and AmB treatment groups was determined using the unpaired *t*-test. The control group was not analyzed statistically because of the small sample number.

RESULTS

Clinical evaluation

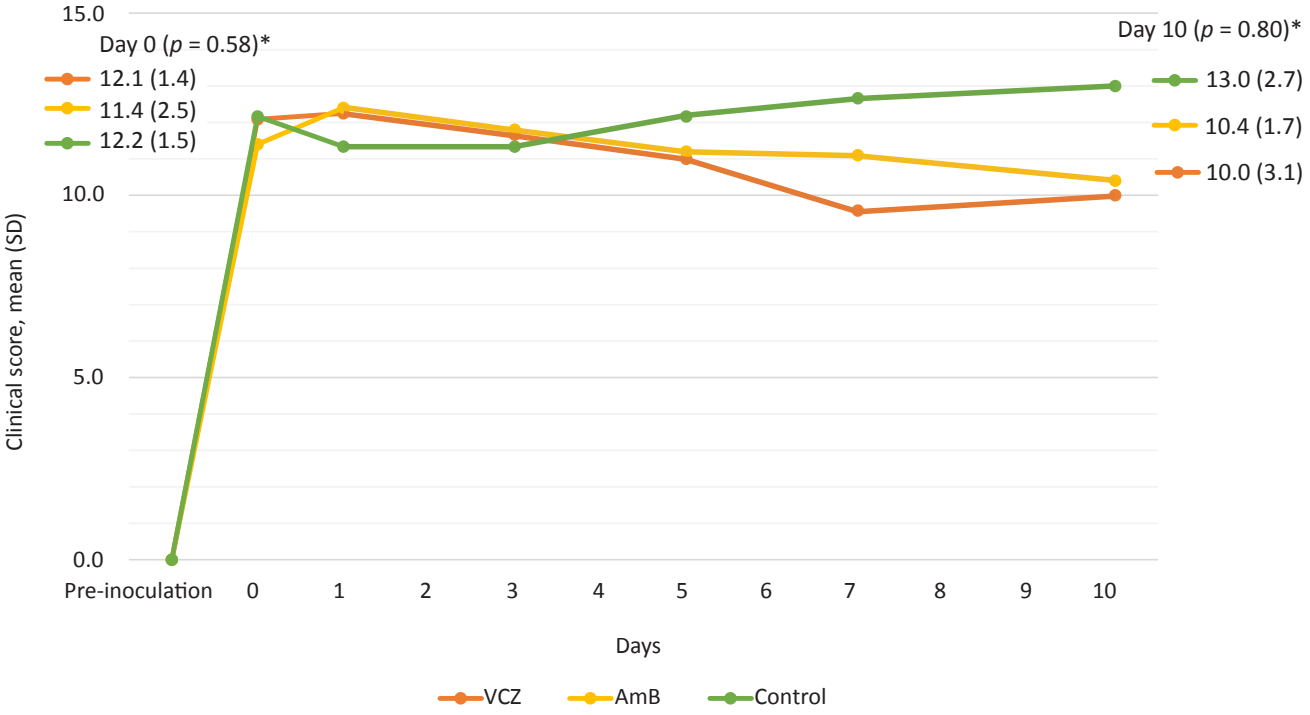
All eyes showed clinical signs of endophthalmitis 24 hours after inoculation. Of the 15 rabbits, one was excluded from the analysis because of contamination on mycological examination. The total sample analyzed in this study was, therefore, 14 eyes. Before treatment initiation, the baseline characteristics were equal among the three groups, as seen in Table 1.

Individual changes of a clinical grading score from each rabbit showed various clinical responses in each group. Clinical improvement was observed in 83.3%, 40%, and 33% of samples of the VCZ, AmB, and control groups, respectively. Clinical improvement was mostly noted in the VCZ and AmB groups, whereas the control group showed the worst deterioration (Figure 1).

Table 1. Baseline characteristic

Characteristics	Group I (VCZ) (n = 6)	Group II (AmB) (n = 5)*	Group III (control) (n = 3)	$p^{\dagger}$
Body weight (gram), mean (SD)	2,713 (179.9)	2,866 (117.2)	2,873 (302.4)	0.14
Total clinical scores 24 hours post-inoculation, mean (SD)	12.1 (1.4)	11.4 (2.5)	12.2 (1.5)	0.58

VCZ=voriconazole; AmB=amphotericin B; SD=standard deviation
*One was dropped out from the AmB group because of contamination; †Unpaired t-test between group I and II



Variable	Δ daily 0 to 10	Δ% mean clinical day 0 to 10	*p**
VCZ	-2.1 (2.8)	-17.4 (22.8)	
AmB	-1.0 (3.2)	-4.2 (29.3)	Δ score $p = 0.56$, % Δ score $p = 0.42$
Control	0.8 (3.1)	8.1 (26.8)	

Figure 1. Daily clinical score and mean clinical score graph of each group. VCZ=voriconazole; AmB=amphotericin B; SD=standard deviation. *Unpaired t-test between Groups I and II

The comparison of clinical scores of all groups at the final evaluation is also shown in Figure 1. No significant difference of total score change was found between the VCZ and AMB groups ($p > 0.05$), but the best clinical response was observed in the VCZ group with 17.4 (22.8%) improvement (Figure 2).

Mycological examination

No fungal structures were detected from all vitreous sample smears on day 10 using wet mount (10% KOH) direct examination. The fungal cultures were positive in 92.8% of samples, showing no growth in only one sample. All growth was identified as *A. flavus* through light microscopy examination using lactophenol cotton blue dye (Figure 3). One sample, however, showed the growth of other *Aspergillus sp.* and was excluded from the analysis. The comparison for fungal quantitative analysis using the CFU count between the two groups is shown in Figure 4a. Significant differences were found between the VCZ and AmB groups, with less CFU in the AmB group ($p < 0.05$). Contrary to the clinical findings, the VCZ group showed the most abundant colony unit growth among other groups.

Histopathologic examination

All eyes were enucleated on day 10, embedded in paraffin, and stained with H&E to examine the intraocular inflammatory reactions to the treatment. Figure 4b shows the mean total histopathologic scores of the three groups. The most severe inflammation was found in the control group with severe abscess formation in the vitreous cavity and retinal structure, followed by the AmB group. Meanwhile, the VCZ group had less severe inflammation with intact retinal structure. However, the difference was not statistically significant ($p > 0.05$).

All samples demonstrated some level of inflammation of the intraocular structures (Figure 5). Of all the three random samples from each group, which had been stained using PAS, only one sample from the control group revealed the presence of hyphae in the intraocular structure.

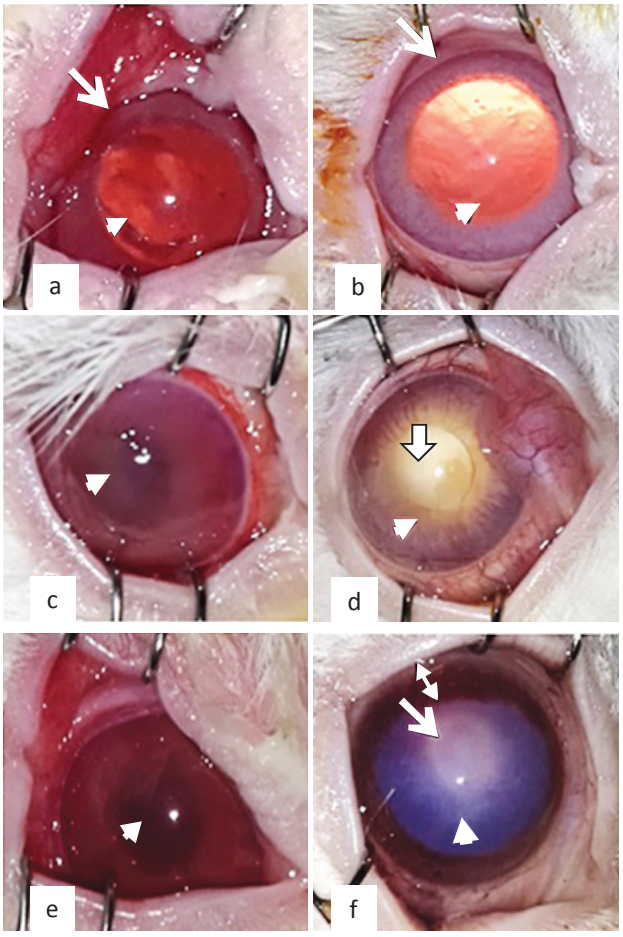


Figure 2. Clinical evaluation of the eyes in each treatment and control group. The VCZ group showed improvement from day 0 (a) to day 10 (b), with less conjunctival chemosis (arrow), corneal edema (arrowhead), anterior chamber inflammation, and clearer vitreous. The AmB group showed clearer cornea (arrowhead) and anterior chamber from day 0 (c) to day 10 (d), but the posterior segment failed to show improvement (bold arrow). The control group showed deterioration from day 0 (e) to day 10 (f), with marked corneal opacity (arrowhead), vast neovascularization (double arrow), fibrin (arrow), and no view of the posterior segment. VCZ=voriconazole; AmB=amphotericin B

DISCUSSION

FE is one of the most fearsome intraocular infections. It can occur as endogenous endophthalmitis, that is, in immunocompromized patients, or as exogenous endophthalmitis due to traumatic ocular injury, keratitis-related endophthalmitis, and postoperative intraocular surgery, such as cataract surgery. It often progresses in destructive nature with poor outcome.[1,2] *A. flavus* is the most common fungal pathogen of postoperative endophthalmitis in Cipto Mangunkusumo Hospital, Jakarta.[4] A similar study has also reported *A. flavus* as the most common pathogen in FE.[5] Hot climate favors the growth and distribution of this fungus, making it the most common environmental contaminant in tropical areas. *A. flavus* invades and destroys intraocular tissue by direct hyphal invasion, toxin, and protease production, along with the added tissue lysis from the immune system in the effort to eliminate this pathogen. These combinations result in vitreous suppuration and massive necrosis of the retinal and choroid structures.[18,19] The best time for therapy was 24 hours after injection because of destructive nature of *A. flavus.*

This study showed a more favorable clinical improvement in the VCZ group (Δ score −2.1 (2.8), 17.4% improvement) compared with the AmB group (Δ score −1.0 (3.2), 4.2% improvement) and worse clinical response in the control group (Δ score 0.8 (3.1), 8.1% deterioration). Although the difference is statistically not significant ($p > 0.05$), the clinical improvement was noted, especially in the anterior segments of the treatment groups. These findings were in accordance with previous reports of the *in vivo* effectiveness of VCZ or AmB in treating FE.[13,19,20] Several *in vitro* studies, however, indicate more favorable results from VCZ compared with AmB, in terms of lower minimal inhibitory concentration needed to eradicate *A. flavus*.[21] This study showed a tendency of lower clinical score in the VCZ group compared with the AmB group, which might be due to the lower concentration of VCZ needed to eliminate *A. flavus*.

The safety of intravitreal injection of 100 µg VCZ and 5 µg AmB has previously been reported in numerous studies.[9,11,22] With the proven safety of both drugs intravitreally, the clinical and anatomical changes that occurred in this study could be assumed to occur solely because of fungal invasion. The multiple frequencies given for the VCZ group were based on the half-life period of VCZ in the rabbit vitreous cavity, which is 2.5 hours, whereas the half-life of AmB is 4.7 days.[23,24]

The milder inflammation of the VCZ group clinically did not go in accordance with the mycological examination. Although direct smear examination with 10% KOH showed no fungal elements in all samples, the quantitative analysis using CFU count showed the greatest number of colony growth in the VCZ group ($p < 0.05$), despite the greatest clinical response in this group. Several factors might have

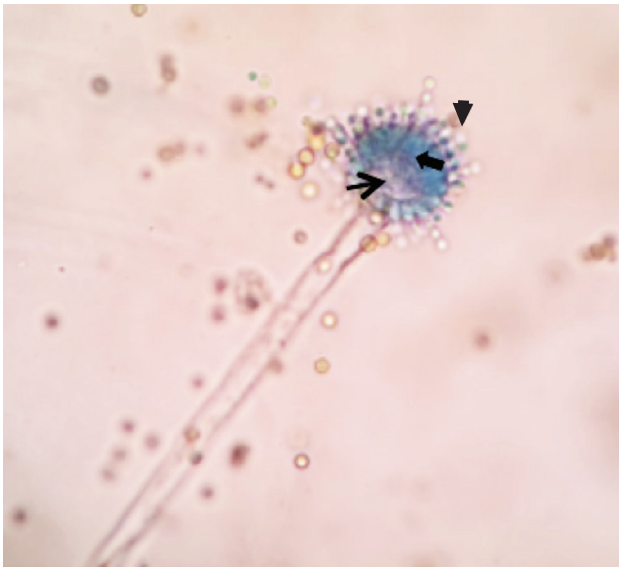


Figure 3. *Aspergillus flavus* morphology using lactophenol cotton blue. A structure of conidiophore with globular vesicle (arrow), surrounded by biseriate phialides (bold arrow), and conidiospores (arrowhead)

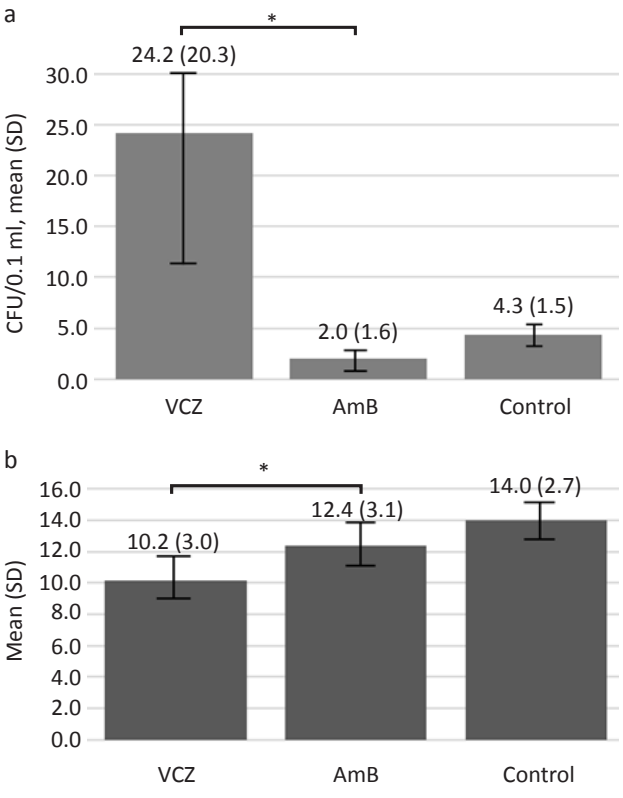


Figure 4. (a) Comparison of fungal quantitative analysis using CFU/0.1 ml on day 10 between each group. Statistically significant difference was found between the VCZ and AmB groups. *Unpaired *t*-test (p = 0.04). (b) Mean histopathologic score on day 10 for each group. Less inflammation score was found in the VCZ group compared with the AmB group *Unpaired *t*-test (p = 0.27). CFU=colony forming unit; SD=standard deviation; VCZ=voriconazole; AmB=amphotericin B

played a role in the discrepancy of the *in vitro* and *in vivo* results shown in this study, including technical factors, conventional culture for diagnostic methods, host immunity, and fungal plasticity.[19,25] Difficulties in the quantification of fungal tissue burden using the CFU count have also been reported, and the results often did not correlate with the actual fungal tissue burden. The suggested newer methods for quantification of fungal tissue burden include tissue chitin detection, DNA-based examination using polymerase chain reaction technique, and antigen detection using the enzyme-linked immunosorbent assay method.[25] A conventional culture method for fungal detection in vitreous has several weaknesses, which include the difficulties in detecting samples with a scarce number of pathogens, or small vitreous sample size, and the tendency of fungal to grow in a cluster, which make them difficult to obtain during a simple vitreous tap procedure.[19]

The clinical evaluation and histopathologic findings in this study showed similar results, with a tendency of milder inflammation in the VCZ group, followed by the AmB group, and most severe inflammation in the control group, although statistically not significant. Eyes with worsening clinical evaluation exhibited more severe histopathologic inflammation with massive polymorphonuclear (PMN) infiltration and *vice versa*. PMN cells hold a vital role in the immune system against fungal infection by directly eliminating fungal hyphae. Therefore, a large number of PMN infiltrations could be seen in all samples, even in the treated groups with clinical improvement. The destructive nature of *A. flavus* also played a role in the relatively inflamed intraocular structures of all samples in this study. The pathogen might have already caused extensive destruction before treatment initiation, and afterward, the residual toxin, inflammatory debris, and waste products might still play a role in the continuation of intraocular tissue destruction.[18]

The major limitation of this study is the small animal sample size. The small sample size might explain the statistically insignificant results shown in this study. Another drawback includes the relatively short time of follow-up, as the long-term effect of treatment could not be demonstrated.

Conclusions

Both intravitreal injection of 100 µg VCZ and 5 µg AmB showed a certain degree of effectiveness

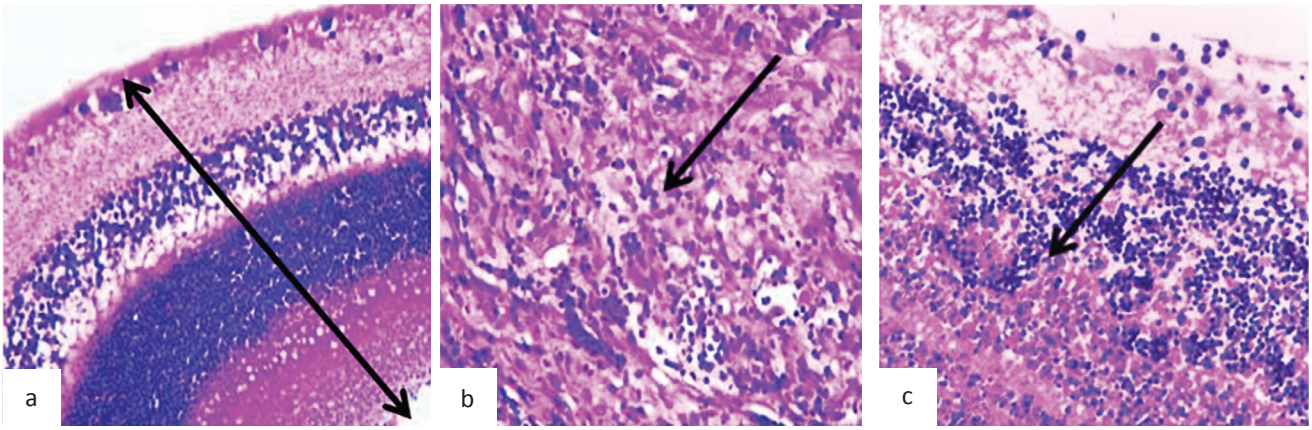


Figure 5. Histopathologic examination on day 10 after treatment. (a) The VCZ group exhibited recognizable retinal structures with edema (double arrow), whereas (b) the AmB and (c) control groups showed unrecognizable retinal structures, forming an abscess-like structure (arrow). VCZ=voriconazole; AmB=amphotericin B

against exogenous *A. flavus* endophthalmitis. VCZ, however, showed a better tendency of clinical improvement and anatomical structure preservation compared with AmB. Further experimental studies to find the optimal treatment regimen for FE are still required. The clinical use of intravitreal VCZ might be considered because of the tendency of better clinical results and its proven intraocular safety profile.

Conflict of Interest

Rianto Setiabudy is one of the editorial board member but was not involved in the review or decision process for the article.

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