Toxicity of Amphotericin B in Rabbit Corneal Epithelial Cells Stored in Optisol™-GS: Corneal Epithelial Cell Morphology and Migration

Running title: Toxicity of AmB in Corneal Epithelium

Kohei Harada[1,2], Hideki Fukuoka[1], Yuriko Ban[1], Yulia Aziza[1], Go Horiguchi[3], Hidetoshi Tanioka[1], Shigeru Kinoshita[4], Masafumi Uematsu[2], Takashi Kitaoka[2] & Chie Sotozono[1]

[1]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

[2]Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

[3]Division of Data Science, The Clinical and Translational Research Center, University Hospital, Kyoto Prefectural University of Medicine, Kyoto, Japan

[4]Department of Frontier Medical Science and Technology for Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

Corresponding author: Chie Sotozono, M.D., Ph.D., Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Hirokoji-agaru, Kawaramachi-dori,

Kamigyo-ku, Kyoto 602-0841, Japan.

Tel: +81-75-251-5578; Fax: +81-75-251-5663; E-mail: csotozon@koto.kpu-m.ac.jp

References: 34; **Figures:** 4; **Tables:** 0

Abstract

Purpose: To evaluate the toxicity of Amphotericin B (AmB) in Optisol™-GS Corneal Storage Media (Bausch & Lomb) on corneal epithelial cell (CEC) morphology and migration ability.

Methods: Sclerocorneal strips were removed from male Japanese white rabbits, and then stored at 4°C in Optisol™-GS containing 0 μg/ml of AmB (control group) and 2.5, 5, 25, and 50 μg/ml of AmB (AmB groups; 4 eyes per group). After 7 days of storage, CEC morphology was evaluated by hematoxylin-eosin staining, immunohistochemical staining (ZO-1), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Moreover, to evaluate CEC migration ability, 3 corneal blocks (6-8 x 3 mm each) from 1 preserved cornea were cultured for 24 hours, and the area of CEC migration (2 mm at the central region) onto the stromal surface was then measured.

Results: At 5, 25, and 50 μg/ml of AmB, deformation and vacuolation of CECs were observed in all preserved corneas. ZO-1 expression was significantly reduced in corneas preserved at AmB concentrations of 25 and 50 μg/ml. TUNEL Labeling Index was significantly increased at AmB concentrations of ≥5 μg/ml. CEC migration was inhibited in a dose-dependent manner at AmB concentrations of 25 and 50 μg/ml compared to the control group.

Conclusions: The addition of AmB to Optisol™-GS can be toxic to CECs and inhibit their migration at a concentration of ≥5 μg/ml. AmB at a concentration of 2.5 μg/ml can be

considered safe for the preservation of donor corneal tissue used in corneal epithelial transplantation surgery.

Keywords: Amphotericin B (AmB) • Optisol™-GS • corneal epithelial cells (CECs) • morphology • epithelial migration

Introduction

In recent years, fungal infection after corneal transplantation has become an issue of significant concern [1-5]. In a 2013 study by the Eye Bank Association of America® (EBAA) Medical Advisory Board Subcommittee on infectious endophthalmitis and keratitis following corneal transplantation, the authors reported fungal infection in 31 of 221,664 corneal transplantations (0.014%) [1]. However, in the 2016 follow-up study by EBAA, the number of fungal infections had increased to 83 of 354,930 corneal transplantations (0.023%) [2]. Reportedly, the most common fungal species responsible for these infections is Candida, and in specific, Candida albicans [4,5]. Although the antifungals Amphotericin B (AmB) [6] and voriconazole [7] are reportedly both effective for inhibiting the growth of Candida, Layer et al. [8] found that at the temperature of 2° to 8°C, AmB was superior to voriconazole. Moreover, AmB is reportedly superior to voriconazole and caspofungin (Cancidas; Merck & Co., Inc., Kenilworth, NJ) in regard to cost-effectiveness [9]. AmB, a polyene macrolide antifungal antibiotic, has a high affinity for ergosterol, a fungal cell membrane component. By binding to these sterols, AmB increases the permeability of the cell membrane and causes the leakage of cytoplasmic components, thus killing the fungus [10,11]. Since AmB also binds to cholesterol, a component of mammalian cell membranes, it is cytotoxic, although with low affinity [12,13].

Optisol™-GS Corneal Storage Media (Bausch & Lomb, Rochester, NY) is the most commonly used medium in the United States for low-temperature preservation of donor

corneas. Optisol™-GS contains the antibiotics gentamicin sulfate (100 μg/ml) and streptomycin sulfate (200 μg/ml), but does not contain antifungal drugs. The common European corneal preservation medium for storage at 31° to 37°C, i.e., an organ culture storage solution, also contains AmB [14-16]. Thus, in recent years, many experiments on corneal preservation have been conducted by adding an antifungal agent to Optisol™-GS [6-8,17-19]. In a study by Tran et al. [17], the findings demonstrated that C. albicans contamination significantly decreased after 6 and 48 hours of incubation with 2.5 μg/ml of AmB in refrigerated Optisol™-GS. Reportedly, this concentration is safe for corneal endothelial cells in terms of mitochondrial function, and cell viability as measured by apoptosis and necrosis [17]. On the other hand, although the level of AmB toxicity to corneal epithelial tissue has been briefly investigated in previous studies, the exact level of toxicity has yet to be fully elucidated [8,19].

Donor corneal epithelium is eventually replaced by recipient epithelial cells after corneal transplantation. Thus, more focus is put on the importance of the corneal endothelium in the corneal preservation process. However, the preservation of corneas lacking corneal epithelium reportedly promotes increased apoptosis of stromal keratocytes [20]. Alternatively, it has also been reported that the time to corneal epithelial healing after corneal transplantation correlates with the degree of donor corneal epithelial loss [21], thus suggesting that not only the corneal endothelium but also the corneal epithelium is important in corneal preservation. Moreover, the proliferative potential of limbal epithelial cells in corneas preserved with

Optisol™-GS decreases with storage time. Thus, the potential of epithelial proliferation or viability is reportedly important in donor corneas used for limbal allograft transplantation and keratoepithelioplasty [22].

Thus, the purpose of this present study was to evaluate the toxicity of AmB on corneal epithelial cells (CECs) stored in refrigerated Optisol™-GS based on histological morphology and CEC migration ability. Moreover, to evaluate CEC migration, we used a newly-developed 2-dimensional evaluation method, i.e., a modified version of the method devised by Nishida et al. [23,24] known as the 'Corneal Epithelial Migration Assay', which involves the use of corneal blocks to simplify and improve the processing and observation technique.

Materials and Methods

Storage medium

AmB (50 mg) in powder form (A9528; Sigma-Aldrich, St. Louis, MO) was diluted with 5 ml of distilled water. A diluted solution was prepared by further diluting 1 ml of the diluted AmB solution (10 mg/ml) with 9 ml of Optisol™-GS. The concentrations of AmB in Optisol™-GS were then each adjusted to 0, 2.5, 5, 25, and 50 μg/ml. In the control group, Optisol™-GS with 0 μg/ml of AmB (i.e., Optisol™-GS without AmB) was used.

Preservation of rabbit corneal tissue

Male Japanese white rabbits (average body weight: 2.5-3 kg) were obtained from Shimizu Laboratory Supplies (Kyoto, Japan). The care and treatment of these animals was conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for Animals Used in Research. The protocols of this study were approved by the Animal Use Committee of Kyoto Prefectural University of Medicine, Kyoto, Japan (Approval No. B2020-102). Briefly, the rabbits were euthanized with an overdose injection of pentobarbital sodium, and corneal buttons with scleral rim were carefully removed using micro tweezers and scissors immediately post sacrifice. Before and after excision, slit-lamp microscopy was used to confirm the absence of CEC damage. A total of 20 corneal strips (4 corneas per group) were then placed on a corneal Independent Viewing Chamber (IVC-12; Bausch & Lomb) with 20 ml of Optisol™-GS containing AmB concentrations of 0, 2.5, 5, 25, and 50 μg/ml, respectively, and then stored at 4°C for 7 days.

Histological staining and immunostaining

For histological staining and immunostaining, the corneal strips preserved under each condition described above were first embedded in OCT compound (Tissue-Tek®; Sakura Finetek Co., Ltd., Tokyo, Japan), and then snap-frozen in liquid nitrogen. Next, cryostat sections (7 μm) were prepared and fixed with 4% paraformaldehyde (PFA). The sections were then subjected to hematoxylin-eosin (HE) staining, immunostaining (ZO-1), and terminal

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

For the HE staining, sections were prepared using a standard method, and the central cornea and peripheral corneal limbus were imaged with an automated fluorescence microscope (BX-63; Olympus Corporation, Tokyo, Japan).

For the immunostaining, the sections were first washed with phosphate-buffered saline (PBS), incubated in PBS containing 0.3% Triton™ X-100 (Thermo Fisher Scientific, Inc., Waltham, MA), a non-ionic surfactant and emulsifier, for 15 minutes, washed twice with PBS for 10 minutes, and blocked with Blocking One (Nakalai Tesque, Inc., Kyoto, Japan) blocking buffer. Next, a 1:100 dilution of the primary ZO-1 monoclonal antibody (1A12; Thermo Fisher Scientific) was applied to the sections, with the sections then being incubated overnight. As a negative control, normal mouse IgG1 (Thermo Fisher Scientific) was used (at the same concentration as the primary antibody). After washing, the cells were incubated with a fluorescence-labeled secondary antibody (Alexa Fluor® 594; Thermo Fisher Scientific), washed again with PBS, then mounted on slides using VECTASHIELD® Antifade Mounting Medium (H-1000-10; Vector Laboratories, Inc., Burlingame, CA). The sections were then covered with a cover glass, and the central portion of the cornea was imaged with a confocal microscope (FV1200 IX83; Olympus). For all staining, 1 slide (3 sections per slide) was used for each corneal sample, with the findings of the three sections then averaged and used for evaluation.

TUNEL labeling assay and cell counting

TUNEL labeling assay was performed using a commercially available kit (DeadEnd™ Fluorometric TUNEL System; Promega Corporation, Madison, WI). Briefly, the 3' end of the fragmented genomic DNA was detected by washing the frozen sections, permeabilizing with 0.2% Triton™ X-100 solution, and incubating in a buffer containing terminal deoxynucleotidyl transferase and fluorescein-12 dUTP. After washing, sections were counterstained with VECTASHIELD® antifade mounting medium and covered with a cover glass. The central portion of the cornea was imaged with the FV1200 IX83 confocal microscope. The 4',6-diamidino-2-phenylindole (DAPI)-positive cells and TUNEL-positive cells were then counted. DAPI (+) and TUNEL (+) cells were counted as apoptotic cells. In addition, those that were DAPI (-) and TUNEL (+) were considered artifacts and not counted [25]. The counting was performed by two ophthalmologists, with the results then averaged. As the TUNEL Labeling Index (LI), the "(Number of positive cells/Total number of nuclei)×100" was calculated and compared for each condition [26]. In addition, the total number of nuclei (DAPI positive) in the CECs was compared for each storage condition to determine the extent of CEC loss.

Corneal epithelial migration assay

A modified version of the Corneal Epithelial Migration Assay observation method reported by Nishida et al. [23,24] was performed as follows. First, three corneal blocks (6-8 × 3 mm each) were prepared from corneal strips preserved under each of the above-described conditions using a razor blade. Reportedly, the corneal block size has no affect on the rate of epithelial migration [23]. Each corneal block was placed epithelial side up in the well of a 12-well cell culture plate (Costar™; Corning Inc., Corning, NY) containing 1 ml of Dulbecco's modified eagle medium and Ham's F12 Nutrient Mixture (DMEM/F-12; Thermo Fisher Scientific). After incubation at 37°C for 24 hours under humidified conditions of 5% CO_2, the corneal blocks were fixed overnight with 4% PFA. Next, the blocks were immersed in PBS for 1 hour, and subjected to HE staining. Briefly, the corneal blocks were immersed in 10% hematoxylin (Funakoshi, Co., Ltd., Tokyo, Japan) for 10 minutes, washed with tap water for 2 minutes, immersed in eosin (Funakoshi) for 5 minutes, washed with tap water for 2 minutes, immersed in 5% ethanol for 5 minutes, and washed with tap water for 2 minutes.

The stained corneal blocks were attached to the back of the glass surface of a glass-bottom dish (Matsunami Glass Industries, Ltd., Osaka, Japan) using the Healon® (1% sodium hyaluronate) Ophthalmic Viscosurgical Device (AMO Japan K.K., Tokyo, Japan). A photograph was then taken with a stereomicroscope (SZX7; Olympus) at two points on the corneal stroma section (longitudinal plane) of each corneal block. The corneal stroma was stained purple with hematoxylin, and the corneal epithelium that had migrated was stained

pink with eosin, thus being respectively distinguished by those colors. As for the measurement area, the top was defined as the top of the corneal block. The CEC migration area extending to the corneal stroma was then measured 2 mm at the block center (Figure 1), with the two migration areas of each block separately averaged.

Statistics and image analysis

The data in this study is reported as mean±standard deviation (SD). Differences between the control group (Optisol™-GS without AmB) and the comparator groups were analyzed using the Dunnett's test, and a *P*-value of < 0.05 was considered statistically significant. The statistical software used in this study was JMP version 14.0.0 (SAS® Institute, Inc., Cary, NC). The area of corneal epithelial migration was measured manually using ImageJ software (United States National Institutes of Health, Bethesda, MD).

Results

Morphological changes in the corneal epithelium

The morphology of CECs preserved for 7 days with Optisol™-GS supplemented with various concentrations of AmB (i.e., 0, 2.5, 5, 25, and 50 μg/ml) was compared. In the AmB 2.5μg/ml group, the corneal epithelium was multilayered, and the morphology of intercellular adhesion and multilayer squamous epithelium was similar to that in the control group (i.e., Optisol™-

GS without AmB). There was also no significant loss of CECs. AmB concentration of ≥ 5 μg/ml caused epithelial damage with vacuolation in the corneal epithelial cytoplasm. AmB at the concentrations of 25 and 50 μg/ml significantly exacerbated vacuolation and nuclear maldistribution. Similar findings were observed in the limbus (Figure 2).

Tissue distribution of ZO-1 as a tight junction (TJ)-related protein in the corneal epithelium was evaluated through immunostaining. In rabbit corneas, ZO-1 is reportedly present in the outermost layer of the epithelium where TJ is present and between wing cells that do not have a TJ structure and basal epithelial cells [27]. The ZO-1 expression and morphology were maintained in the outermost layer of the corneal epithelium and between the wing cells and basal epithelial cells in AmB concentrations of up to 5 μg/ml, similar to the control group; however, a marked disappearance of ZO-1-positive areas was observed at AmB concentrations of 25 and 50 μg/ml (Figure 2).

TUNEL labeling assay and cell counting

TUNEL LI significantly increased starting from 5 μg/ml at 48.6 ± 8.72% ($P = 0.0327$) compared to the control (33.4 ± 5.84%), with further significant increases at 25 and 50 μg/ml to 59.9 ± 8.96% ($P = 0.0004$) and 60.0 ± 7.34% ($P = 0.0004$), respectively. There were no significant differences between the control group and the 2.5 μg/ml AmB group (Figure 3).

Moreover, when the total number of nuclei (DAPI positive) in the CECs was compared,

there were no significant differences compared to the control group (218.1 ± 7.38 cells), even at high AmB storage conditions, such as concentrations of 25 and 50 µg/ml, where TUNEL LI significantly increased (Figure 3).

Corneal epithelial migration ability

Epithelial migration ability was evaluated for 24 hours in corneal blocks after 7 days of storage under each of the above-described conditions.

The distance of epithelial migration was not necessarily constant, even within each block, as shown in Figure 1 and Figure 4. With 2.5 µg/ml and 5 µg/ml of AmB, the mean area of epithelial migration was 1.12 ± 0.17 mm^2 ($P = 0.9931$) and 0.93 ± 0.21 mm^2 ($P = 0.3188$), respectively, while that of the control was 1.16 ± 0.18 mm^2 in comparison. The area was 0.73 ± 0.27 mm^2 ($P = 0.0244$) and 0.66 ± 0.12 mm^2 ($P = 0.0095$) for the concentrations of 25 and 50 µg/ml, respectively, thus demonstrating significant differences at those concentrations (Figure 4). There were no significant differences compared to the control in the 2.5 and 5 µg/ml groups.

Discussion

The results of this study demonstrated that 2.5 µg/ml of AmB added to Optisol™-GS was safe for CECs, however, concentrations of ≥ 5 µg/ml can be toxic to CECs and inhibit CEC

migration in rabbit corneas. Furthermore, even the 2.5 μg /ml of AmB contained in refrigerated Optisol™-GS exhibits sufficient antifungal activity against fungi such as C. albicans [17]. Moreover, an AmB concentration of 2.5 ug/ml is reportedly sufficient for inhibiting the formation of resistance to anti-fungus drugs, which sometimes can be induced when lower concentrations are used [28]. In recent years, due to the increase in fungal infections after corneal transplantation, the addition of antifungal drugs, i.e., AmB, in particular, to corneal storage medium has attracted broad attention. Several studies have reported on the concentration of AmB in Optisol™-GS, which exerts antifungal effects on Candida species, and evaluations have been conducted on its toxicity on corneal endothelial cells [6,8,17-19]. Layer et al. [8] reported that in corneal epithelium, no damage was observed up to an AmB concentration of 40 μg/ml. However, in that study, the corneal epithelium was observed only by slit-lamp microscopy, and no detailed evaluations were made. Thus, and to the best of our knowledge, this is the first study to evaluate the toxicity of AmB on CECs in terms of histological morphology and corneal epithelial migration ability. In addition, in the previous studies investigating corneal preservation, the focus was mainly on corneal endothelial cells. However, the corneal epithelium is also important, as donor corneas are used for epithelial transplantation, e.g., limbal allograft transplantation [29,30] and keratoepithelioplasty [31].

In this present study, the toxicity of AmB on rabbit CECs stored in Optisol™-GS was evaluated based on histological morphology and corneal epithelial migration. The results in

rabbits showed good epithelial morphology in the control group and the 2.5 μg/ml AmB group based on HE staining of pathological morphology, even after 7 days of storage, while damage such as epithelial vacuoles was observed when the AmB concentration was ≥ 5 μg/ml. In immunostaining using ZO-1, a TJ-associated protein, the disappearance of ZO-1-positive areas was seen in AmB concentrations of ≥ 25 μg/ml. Means et al. [32] reported that storing corneas in Optisol™-GS caused epithelial damage, including marked intracellular edema and epithelial damage, in the corneal epithelium on days 7-10, thus suggesting that AmB accelerates corneal epithelial damage, especially at the concentration of ≥ 5.0 μg/ml.

TUNEL LI in the TUNEL staining showed significantly increased apoptosis with AmB at concentrations of ≥ 5 μg/ml. According to previous reports, the number of apoptosis-positive cells increased with the corneal-epithelium storage time in Optisol™-GS at 4°C [22,33]. However, in those studies, only the number of apoptosis-positive cells per section was measured. To the best of our knowledge, there have been no previous reports on the preservation of rabbit corneal epithelium by calculating TUNEL LI, i.e., the proportion of apoptosis-positive cells to the total number of CECs, which was revealed in this present study. In addition, our findings showed no differences in the comparison of the total number of nuclei (DAPI positive) in CECs between all groups. Based on this finding, even when stored at high concentrations of AmB for 7 days, CECs are not sloughed; instead, vacuoles are generated in the cytoplasm, and nuclei are maldistributed at the end of storage.

Epithelial migration was mildly inhibited by AmB at the concentration of 5 μg/ml, and significantly inhibited by AmB at the concentration of ≥ 25 μg/ml. The corneal epithelium maintains its morphology by migrating from the cell division performed by basal cells to the surface layer [34]. In HE staining, corneal epithelial basal cells remained almost normal at an AmB concentration of 5 μg/ml compared to 25 and 50 μg/ml. Therefore, it is conceivable that the AmB concentration of 5 μg/ml did not cause a strong enough epithelial elongation impairment to make a significant difference.

For the evaluation of corneal epithelial migration ability, we modified the method previously reported by Nishida et al. [23,24]. In the conventional method, the distance of migration of the epithelium at both ends of the section is measured one-dimensionally. In addition, it was not feasible to observe the entire block. Thus, we modified the method so that we could observe the entire block two-dimensionally. The pink stained area, which is the migrated CECs from the original epithelium, can be observed clearly via the stereomicroscope. This assay was easy to perform, and provided for a more reliable evaluation of the migration ability.

It should be noted that this current study did have some limitations. First, the experiments performed in this study involved the use of rabbit corneas, as Japanese law prohibits the use of domestic human donor corneas in academic research. Second, the number of vacuoles and the ZO-1 expression in the histological experiments were not quantitatively

evaluated. The findings in this study reveal that an AmB concentration of ≥ 5 μg/ml in Optisol™-GS may affect CEC morphology and migration ability. It should be noted that there are numerous corneal transplantation techniques, such as penetrating keratoplasty, epithelial transplantation, endothelial transplantation, etc. However, the method used for corneal preservation has yet to be standardized globally. Thus, the toxicity of AmB needs to be further evaluated, not only in regard to corneal endothelial cells, but also CECs. Our findings showed that AmB at the concentration of 2.5 μg/ml can be considered safe and effective for the preservation of donor corneal tissue used for corneal epithelial transplantation surgery. However, for a more detailed and precise evaluation of AmB toxicity, further studies evaluating morphological, functional, and clinical changes in human corneal grafts may be necessary.

Acknowledgments

The authors wish to thank John Bush for reviewing the manuscript.

Conflicts of interest

No commercial relationships. No conflicting relationship exists for any author.

Declaration of interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Funding

Funding Information is not available..

Data availability statement

The data that support the findings of this study are available from the first author, K.H. , upon reasonable request.

References

1. Aldave AJ, DeMatteo J, Glasser DB, Tu EY, Iliakis B, Nordlund ML, Misko J, Verdier DD, Yu F. Report of the Eye Bank Association of America medical advisory board subcommittee on fungal infection after corneal transplantation. Cornea. 2013;32(2):149-154.

2. Edelstein SL, DeMatteo J, Stoeger CG, Macsai MS, Wang CH. Report of the Eye Bank Association of America Medical Review Subcommittee on Adverse Reactions Reported From 2007 to 2014. Cornea. 2016;35(7):917-926.

3. Hassan SS, Wilhelmus KR. Eye-banking risk factors for fungal endophthalmitis compared with bacterial endophthalmitis after corneal transplantation. Am J Ophthalmol. 2005;139(4):685-690.

4. Merchant A, Zacks CM, Wilhelmus K, Durand M, Dohlman CH. Candidal endophthalmitis after keratoplasty. Cornea. 2001;20(2):226-229.

5. Vislisel JM, Goins KM, Wagoner MD, Schmidt GA, Aldrich BT, Skeie JM, Reed CR, Zimmerman MB, Greiner MA. Incidence and outcomes of positive donor corneoscleral rim fungal cultures after keratoplasty. Ophthalmology. 2017;124(1):36-42.

6. Duncan K, Parker J, Hoover C, Lindquist TD, Jeng BH. Efficacy of amphotericin B against fusarium and aspergillus in corneal storage medium. Eye Contact Lens. 2018;44(6):390-392.

7. Ritterband DC, Shah MK, Meskin SW, Seedor JA, Koplin RS, Perez W, Yang R, Hu DN, Dahl P. Efficacy and safety of voriconazole as an additive in Optisol GS: a preservation medium for corneal donor tissue. Cornea. 2007;26(3):343-347.

8. Layer N, Cevallos V, Maxwell AJ, Hoover C, Keenan JD, Jeng BH. Efficacy and safety of antifungal additives in Optisol-GS corneal storage medium. JAMA Ophthalmol. 2014;132(7):832-837.

9. Gibbons A, Leung EH, Yoo SH. Cost-effectiveness of antifungal supplementation of corneal cold storage media. Ophthalmology. 2020;127(5):582-588.

10. Andreoli TE. The structure and function of amphotericin B-cholesterol pores in lipid bilayer membranes. Ann N Y Acad Sci. 1974;235(0):448-468.

11. de Kruijff B, Gerritsen WJ, Oerlemans A, Demel RA, van Deenen LL. Polyene antibiotic-sterol interactions in membranes of Acholeplasma laidlawii cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by the polyene antibiotics. Biochim Biophys Acta. 1974;339(1):30-43.

12. Thanki K, Prajapati R, Sangamwar AT, Jain S. Long chain fatty acid conjugation remarkably decreases the aggregation induced toxicity of amphotericin B. Int J Pharm. 2018;544(1):1-13.

13. Wang J, Ma Y, Hou S, Miao Z, Ma Q. Interaction of amphotericin B and saturated or unsaturated phospholipid monolayers containing cholesterol or ergosterol at the air-

water interface. Biophys Chem. 2020;258:106317.

14. Armitage WJ. Preservation of human cornea. Transfus Med Hemother. 2011;38(2):143-147.

15. Pels E, Schuchard Y. Organ-culture preservation of human corneas. Doc Ophthalmol. 1983;56(1-2):147-153.

16. Frueh BE, Böhnke M. Corneal grafting of donor tissue preserved for longer than 4 weeks in organ-culture medium. Cornea. 1995;14(5):463-466.

17. Tran KD, Aldrich BT, D'Amato Tóthová J, Skeie JM, Kondratick CM, Giurgola L, Gatto C, Reed CR, Schmidt GA, Terry MA, et al. Efficacy and Safety of Various Amphotericin B Concentrations on Candida albicans in Cold Storage Conditions. Cornea. 2020;39(1):110-117.

18. Tran D, Dhaliwal D, Kamyar R, Jhanji V, Kowalski RP. Effect of Optisol supplementation with 0.255 μg/mL amphotericin B on elimination of yeast at 5°C. Cornea. 2019;38(7):901-904.

19. Duncan K, Parker J, Hoover C, Jeng BH. The effect of light exposure on the efficacy and safety of amphotericin B in corneal storage media. JAMA Ophthalmol. 2016;134(4):432-436.

20. Chang SW, Wang YH, Pang JH. The effects of epithelial viability on stromal keratocyte apoptosis in porcine corneas stored in Optisol-GS. Cornea. 2006;25(1):78-84.

21. Meyer RF, Bobb KC. Corneal epithelium in penetrating keratoplasty. Am J Ophthalmol. 1980;90(2):142-147.

22. Li C, Dong N, Wu H, Dong F, Xu Y, Du H, He H, Liu Z, Li W. A novel method for preservation of human corneal limbal tissue. Invest Ophthalmol Vis Sci. 2013;54(6):4041-4047.

23. Nishida T, Nakagawa S, Awata T, Ohashi Y, Watanabe K, Manabe R. Fibronectin promotes epithelial migration of cultured rabbit cornea in situ. J Cell Biol. 1983;97(5 Pt 1):1653-1657.

24. Nakamura M, Nishida T, Ofuji K, Reid TW, Mannis MJ, Murphy CJ. Synergistic effect of substance P with epidermal growth factor on epithelial migration in rabbit cornea. Exp Eye Res. 1997;65(3):321-329.

25. Xing D, Bonanno JA. Hypoxia preconditioning protection of corneal stromal cells requires HIF1alpha but not VEGF. Mol Vis. 2009;15:1020-1027.

26. Duan WR, Garner DS, Williams SD, Funckes-Shippy CL, Spath IS, Blomme EA. Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. J Pathol. 2003;199(2):221-228.

27. Sugrue SP, Zieske JD. ZO1 in corneal epithelium: association to the zonula occludens and adherens junctions. Exp Eye Res. 1997;64(1):11-20.

28. Dalhoff A. Does the use of antifungal agents in agriculture and food foster polyene resistance development? A reason for concern. J Glob Antimicrob Resist. 2018;13:40-48.

29. Tseng SC, Tsai RJ. Limbal transplantation for ocular surface reconstruction--a review. Fortschr Ophthalmol. 1991;88(3):236-242.

30. Tsai RJ, Tseng SC. Human allograft limbal transplantation for corneal surface reconstruction. Cornea. 1994;13(5):389-400.

31. Thoft RA. Keratoepithelioplasty. Am J Ophthalmol. 1984;97(1):1-6.

32. Means TL, Geroski DH, L'Hernault N, Grossniklaus HE, Kim T, Edelhauser HF. The corneal epithelium after optisol-GS storage. Cornea. 1996;15(6):599-605.

33. Komuro A, Hodge DO, Gores GJ, Bourne WM. Cell death during corneal storage at 4 degrees C. Invest Ophthalmol Vis Sci. 1999;40(12):2827-2832.

34. Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. Invest Ophthalmol Vis Sci. 1983;24(10):1442-1443.

Figure Legends

Figure 1. Diagram and images illustrating the corneal epithelial migration assay. A) Schematic diagram of the corneal block (modified from Reference 21). B) Stereoscopic microscopic findings of corneal blocks cultured for 24 hours. The corneal stroma was stained purple with hematoxylin, and the corneal epithelium that migrated was stained pink with eosin. C) The area of the corneal epithelium that migrated into the corneal stroma measured 2 mm at the center of the corneal block using ImageJ software. D) Expanded image of Figure C.

Figure 2. Hematoxylin-eosin (HE) staining and ZO-1immunostaining of the central portion of the corneal epithelium after storage for 7 days under each condition described in the text (n = 4). HE staining showed good epithelial morphology and no significant loss of epithelial cells in the control group and the Amphotericin (AmB) concentration of 2.5 μg/ml. AmB at the concentration 5 μg/ml caused epithelial damage with vacuolation in the corneal epithelial cytoplasm. At the AmB concentrations of 25 and 50 μg/ml, a marked aggravation of vacuolation (→) and nuclear maldistribution (⇨) was observed. Similar findings were seen in the limbus. In immunostaining, the ZO-1 expression and morphology were maintained in the outermost layer of the corneal epithelium and between the wing cells and basal epithelial cells up to an AmB concentration of 5 μg/ml, as in the control group. However, a marked disappearance of ZO-1-positive areas was observed at the AmB concentrations of 25 and 50

μg/ml.

Figure 3. Apoptosis in the central portion of the corneal epithelium after 7 days of storage under each condition and a comparison of the number of DAPI-positive nuclei in corneal epithelial cells (CECs) (n = 4). A) TUNEL labeling index (LI) significantly increased starting from 5 μg/ml AmB compared to the control. There were no significant differences between the control group and the AmB 2.5 μg/ml group (* $P < 0.05$, ** $P < 0.0005$). B) Comparisons of the total number of nuclei (DAPI positive) in CECs showed no significant differences from the control group, even at high AmB storage conditions such as 25 and 50 μg/ml where TUNEL LI significantly increased.

Figure 4. Corneal epithelial migration ability after 7 days of storage under each condition (n = 4). The area of epithelial migration was 0.93 ± 0.21 mm^2 in the AmB 5 μg/ml group and 0.73 ± 0.27 mm^2 and 0.66 ± 0.12 mm^2 in the AmB 25 μg/ml and 50 μg/ml groups, respectively, compared to the control group at 1.16 ± 0.18 mm^2, thus showing a significant difference (* $P < 0.05$, ** $P < 0.01$). The AmB 2.5μg/ml group was 1.16 ± 0.17 mm^2, which was not significantly different from that in the control group. Scale bar = 2 mm.

Figure 1

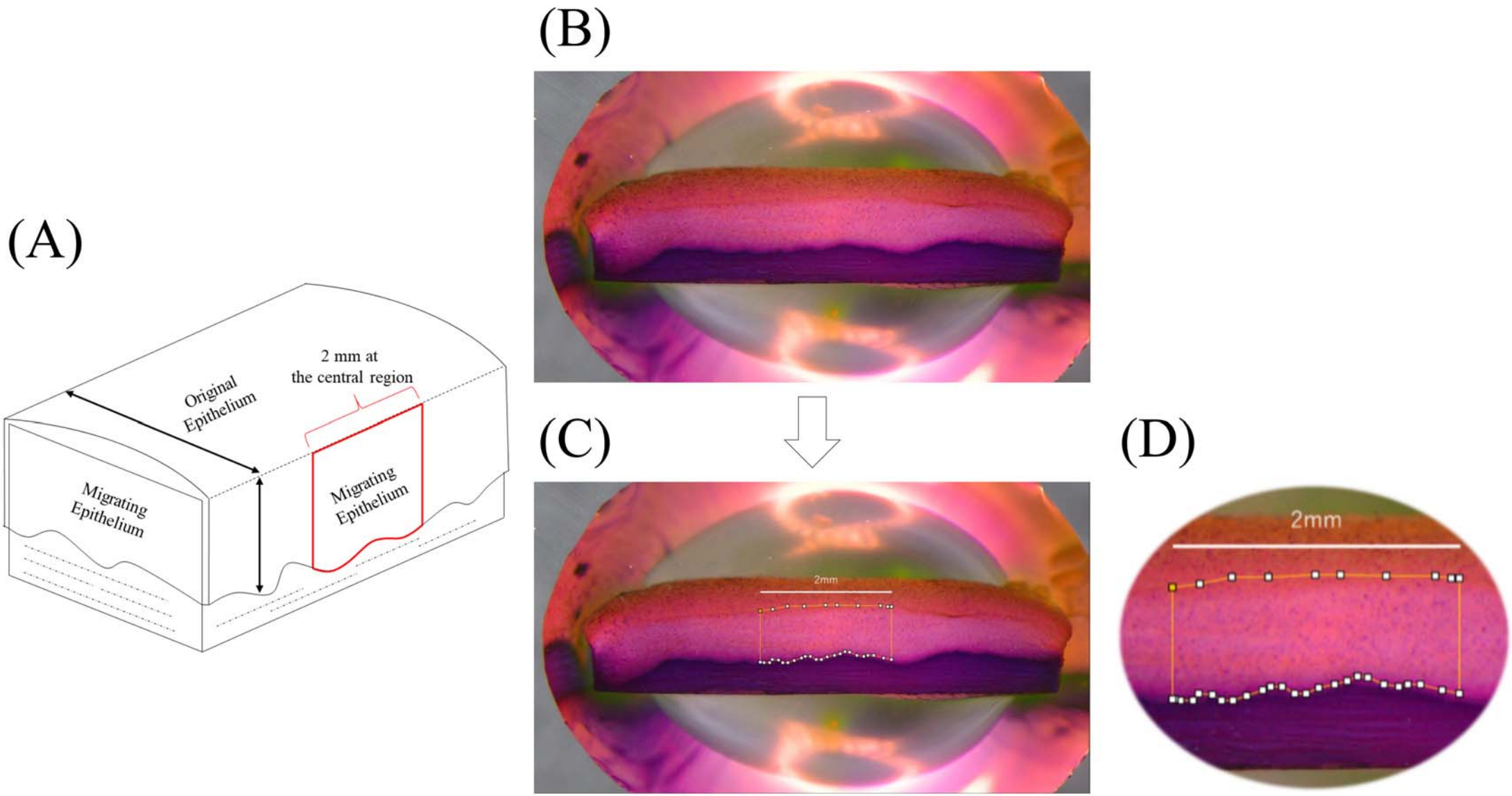

(A)
Original Epithelium
2 mm at the central region
Migrating Epithelium
Migrating Epithelium
(B)
(C)
2mm
(D)
2mm

Figure 2

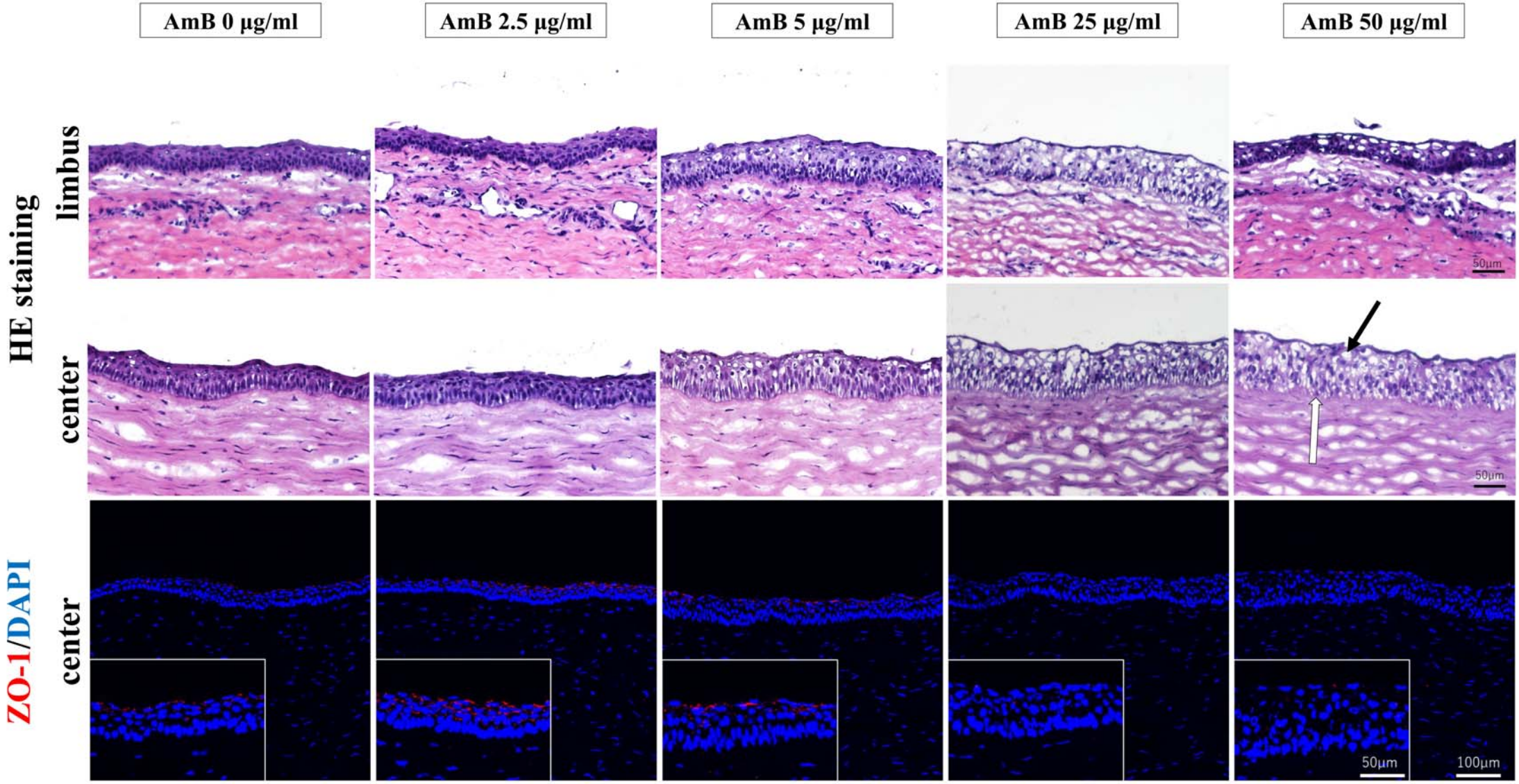

AmB 0 μg/ml
AmB 2.5 μg/ml
AmB 5 μg/ml
AmB 25 μg/ml
AmB 50 μg/ml
HE staining
limbus
center
ZO-1/DAPI
center
50μm
50μm
50μm
100μm

Figure 3

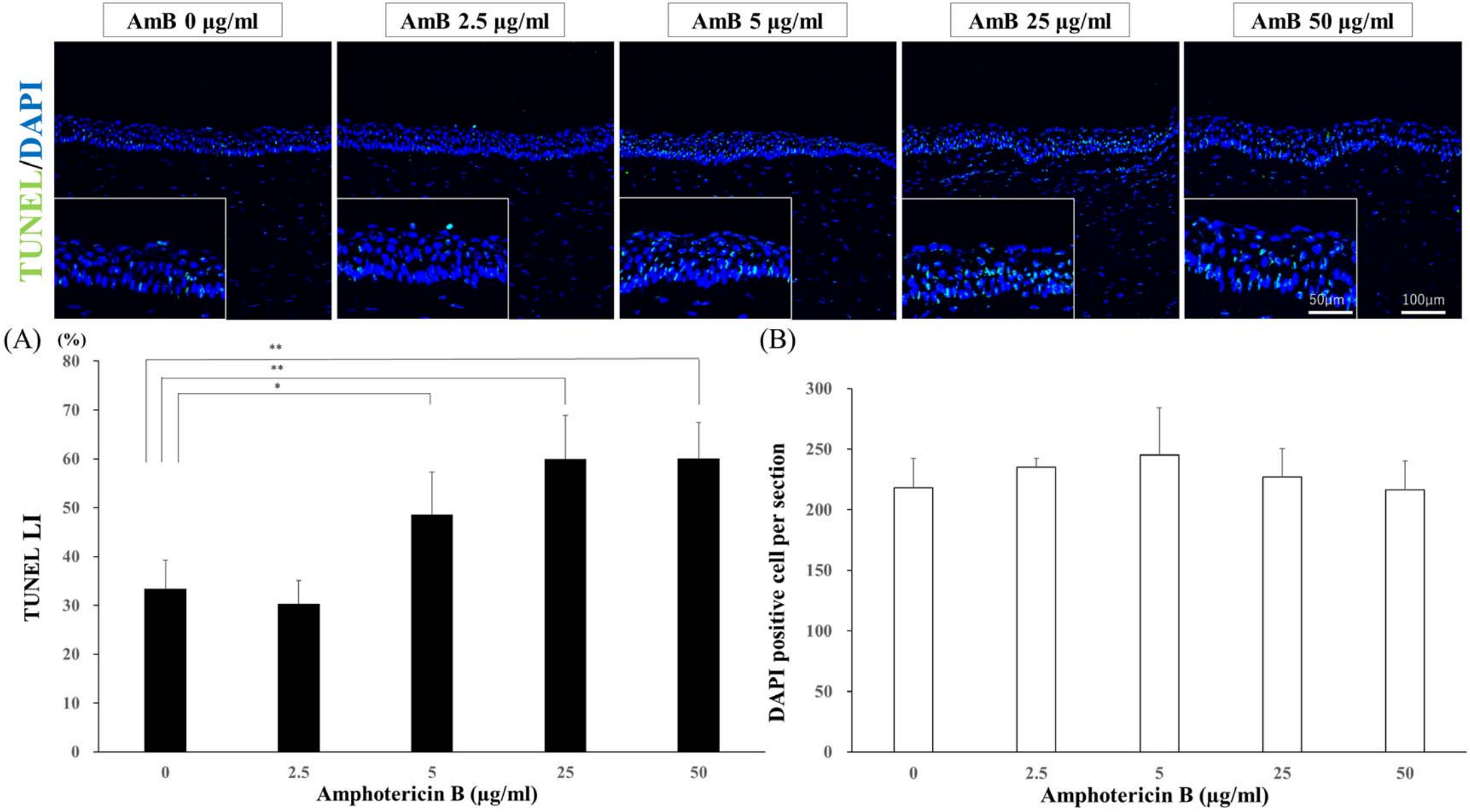
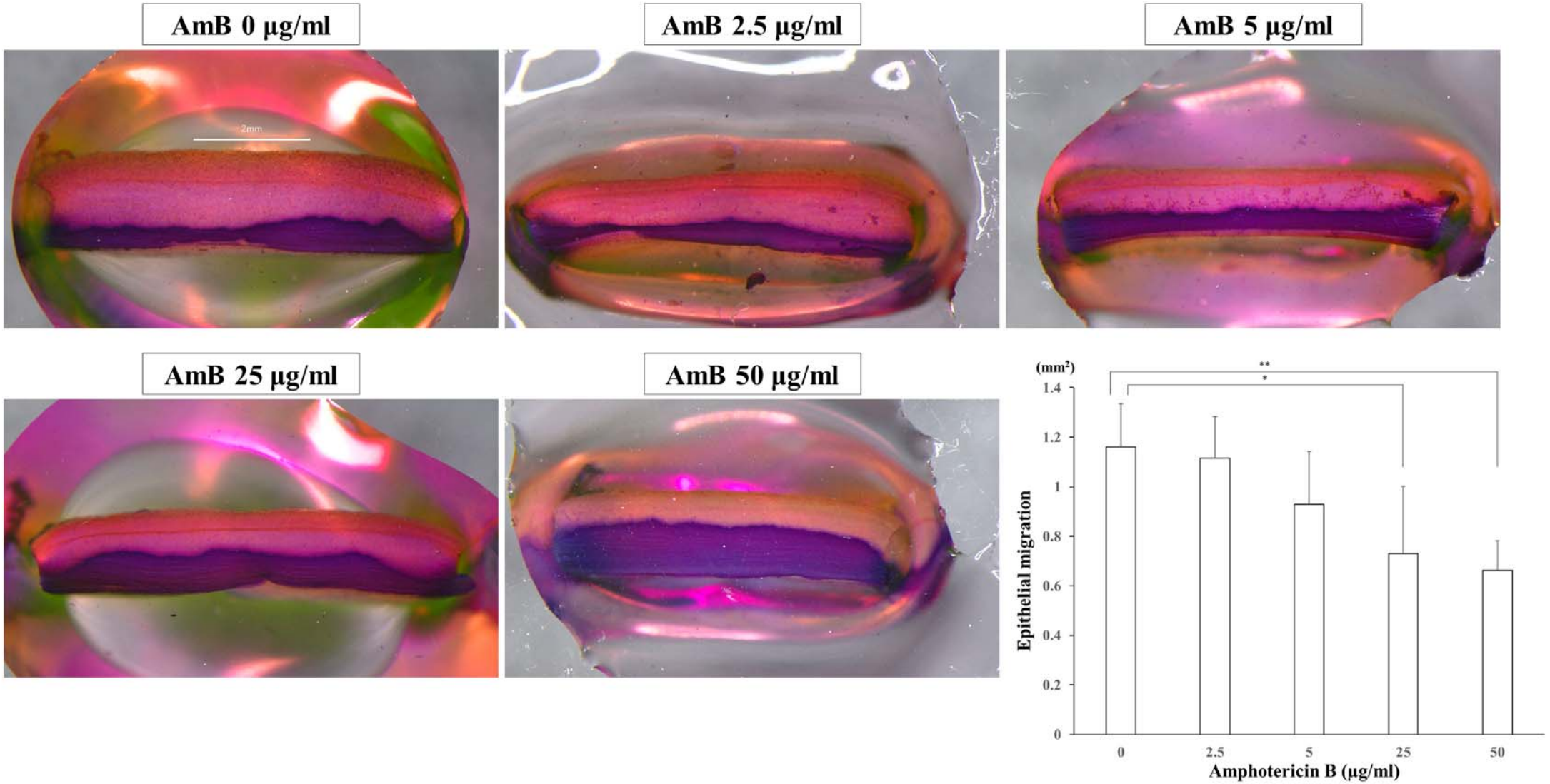

AmB 0 μg/ml
AmB 2.5 μg/ml
AmB 5 μg/ml
AmB 25 μg/ml
AmB 50 μg/ml
TUNEL/DAPI
50μm
100μm
(A)
(%)
TUNEL LI
Amphotericin B (μg/ml)
(B)
DAPI positive cell per section
Amphotericin B (μg/ml)

Figure 4


AmB 0 µg/ml
2mm
AmB 2.5 µg/ml
AmB 5 µg/ml
AmB 25 µg/ml
AmB 50 µg/ml
(mm²)
**
*
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Epithelial migration
0
2.5
5
25
50
Amphotericin B (µg/ml)