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Original article

# How to prevent contamination with *Candida albicans* during the fabrication of transplantable oral mucosal epithelial cell sheets

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ABSTRACT

We have utilized patients' own oral mucosa as a cell source for the fabrication of transplantable epithelial cell sheets to treat limbal stem cell deficiency and mucosal defects after endoscopic submucosal dissection of esophageal cancer. Because there are abundant microbiotas in the human oral cavity, the oral mucosa was sterilized and 40 μg/mL gentamicin and 0.27 μg/mL amphotericin B were added to the culture medium in our protocol. Although an oral surgeon carefully checked each patient's oral cavity and although candidiasis was not observed before taking the biopsy, contamination with *Candida albicans* (*C. albicans*) was detected in the conditioned medium during cell sheet fabrication. After adding 1 μg/mL amphotericin B to the transportation medium during transport from Nagasaki University Hospital to Tokyo Women's Medical University, which are 1200 km apart, no proliferation of *C. albicans* was observed. These results indicated that the supplementation of transportation medium with antimycotics would be useful for preventing contamination with *C. albicans* derived from the oral mucosa without hampering cell proliferation.



*Abbreviations: C. albicans*, *Candida albicans*; DMEM, Dulbecco's modified Eagle's medium.

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Cultured oral mucosal epithelial cells have been utilized for sympatric and ectopic transplantation to reconstruct stratified epithelia such as the oral mucosa, skin, and cornea [1–3]. After optimizing culture medium containing autologous serum for fabricating autologous oral mucosal epithelial cell sheets, we have treated an esophageal ulcer resulting from endoscopic mucosal dissection of a mucosal tumor by performing endoscopic transplantation of autologous oral mucosal epithelial cell sheets fabricated on temperature-responsive cell culture surfaces to promote wound healing and prevent stenosis [4–6].

Because the human oral cavity contains abundant microbiota, biopsies of oral mucosa are treated with povidone-iodine. Furthermore, biopsies are stored in Dulbecco's modified Eagle's medium (DMEM) supplemented with 86 μg/mL ampicillin-sulbactam (Unasyn-S; Pfizer, NY, USA) and 100 μg/mL streptomycin (Meiji Seika Pharma, Tokyo, Japan) during transport from the oral surgery department to the cell culture facility. Moreover, the tissue is treated with povidone-iodine in the cell culture facility and is treated with dispase in DMEM including the same concentrations of ampicillin-sulbactam and streptomycin for epithelium separation. In addition, we add 40 μg/mL gentamicin (Gentacin; Schering-Plough, NJ, USA) and 0.27 μg/mL amphotericin B (Fungizone; Bristol-Myers Squibb, NY, USA) to the culture medium to maintain a sterile environment. Therefore, we have not experienced bacterial or fungal contamination in 8 biopsies from healthy volunteer donors in a preclinical study or in 10 biopsies from patients suffering from esophageal cancer treated at Tokyo Women's Medical University [6,7]. We have performed another clinical research study to examine the safety of long-distance transport of fabricated cell sheets between Tokyo Women's Medical University and Nagasaki University Hospital, which are approximately 1200 km apart, with transport taking 5–7 h by air and train. The protocol for oral mucosal epithelial cell sheet transplantation into patients was approved by the Ethical Committees and Internal Review Boards of Nagasaki University and Tokyo Women's Medical University. Approval of this clinical study by the Health, Labour and Welfare Ministry was gained on March 29th, 2013. Unfortunately, we experienced contamination with a yeast-like fungus in the culture supernatant of a patient's oral mucosal epithelial cells, so we abandoned the fabricated cell sheets for transplantation. We then performed sterilization tests to identify the source of the contamination and the strain of the fungus. Supernatants from each sample were cultured in soybean-casein digest broth (Wako Pure Chemical Industries, Osaka, Japan) and alternative thioglycollate medium (Wako Pure Chemical Industries). The strain of the cultured fungus was identified using CHROMagar Candida (Becton, Dickinson and Company, NJ, USA) and API 20C AUX (bioMérieux, Lyon, France). The obtained results revealed that the patient's oral mucosa was the source of *C. albicans* (*C. albicans*), as described below (Table 1). The oral mucosal tissue appeared macroscopically healthy (Fig. 1A), and there was no *Candida* antigen or infection with *C. albicans* in the patient's serum, which was added to the culture medium (Table 1). In addition, the cultured oral mucosal epithelial cells exhibited normal cell morphology (Fig. 1B,C). However, contaminating *C. albicans* and hyphal formation were detected during epithelial cell culture (Fig. 1D,E). It should be noted that hyphal formation by *C. albicans* was inhibited under anaerobic conditions [8].

We then tested the susceptibility of the *C. albicans* strain obtained from the conditioned medium and the oral surface of the patient to antimycotic agents using a commercially prepared colorimetric microdilution panel (ASTY; Kyokuto Pharmaceutical Industrial, Tokyo, Japan) [9]. The proliferation of the strain was completely inhibited by 0.5 μg/mL amphotericin B. In comparison, in previous susceptibility testing, the proliferation of nearly all *Candida* species was inhibited by 1.0 μg/mL amphotericin B [10], and a higher concentration of amphotericin B often hampers mammalian cell proliferation [11]. Therefore, we changed our protocol for the transport of oral mucosal biopsies from Nagasaki University Hospital to Tokyo Women's Medical University. The DMEM used for the transportation was supplemented with 1.0 μg/mL amphotericin B, and the concentration of amphotericin B in the culture medium was kept at 0.27 μg/mL, with no modification.

It took approximately 6 h to transport the biopsy by air and train, and then the transported biopsy was subjected to harvesting of the oral mucosal epithelial cells using dispase treatment for 2 h at 37 °C in DMEM supplemented with the same concentration of amphotericin B. As a result, no contamination with *C. albicans* was observed in the supernatant of the culture medium used for the fabrication of transplantable epithelial cell sheets from the same

**Table 1**
The results of quality control tests.

| Sample | Items | | Result |
|---|---|---|---|
| Cell culture supernatant (1st trial)[a] | Sterilization test | Bacteria | Negative |
| | | Fungi | *Candida albicans* |
| | Mycoplasmal culture | | Negative |
| | Mycoplasma test (PCR)[b] | | Negative |
| | Endotoxin | | 0.062 EU/mL |
| Reagents for cultivation | Sterilization test | Bacteria | Negative |
| | | Fungi | Negative |
| Serum (patient) | Sterilization test | Bacteria | Negative |
| | | Fungi | Negative |
| | | Candida antigen | Negative |
| Oral surface (patient) | Sterilization test | Fungi | *Candida albicans* |
| Oral surface (operator 1) | Sterilization test | Fungi | Negative |
| Oral surface (operator 2) | Sterilization test | Fungi | Negative |
| Cell culture supernatant (2nd trial)[a] | Sterilization test | Bacteria | Negative |
| | | Fungi | Negative |
| | | Mycoplasmal culture | Negative |
| | | Mycoplasma test (PCR)[b] | Negative |
| | | Endotoxin | 0.136 EU/mL |
| Oral surface (patient) | Sterilization test | Fungi | *Candida albicans* |

[a] Cell culture supernatants were routinely used for quality control tests.
[b] PCR for detecting *Mycoplasma pneumoniae* was performed in accordance with method shown by Jensen JS et al. [12].

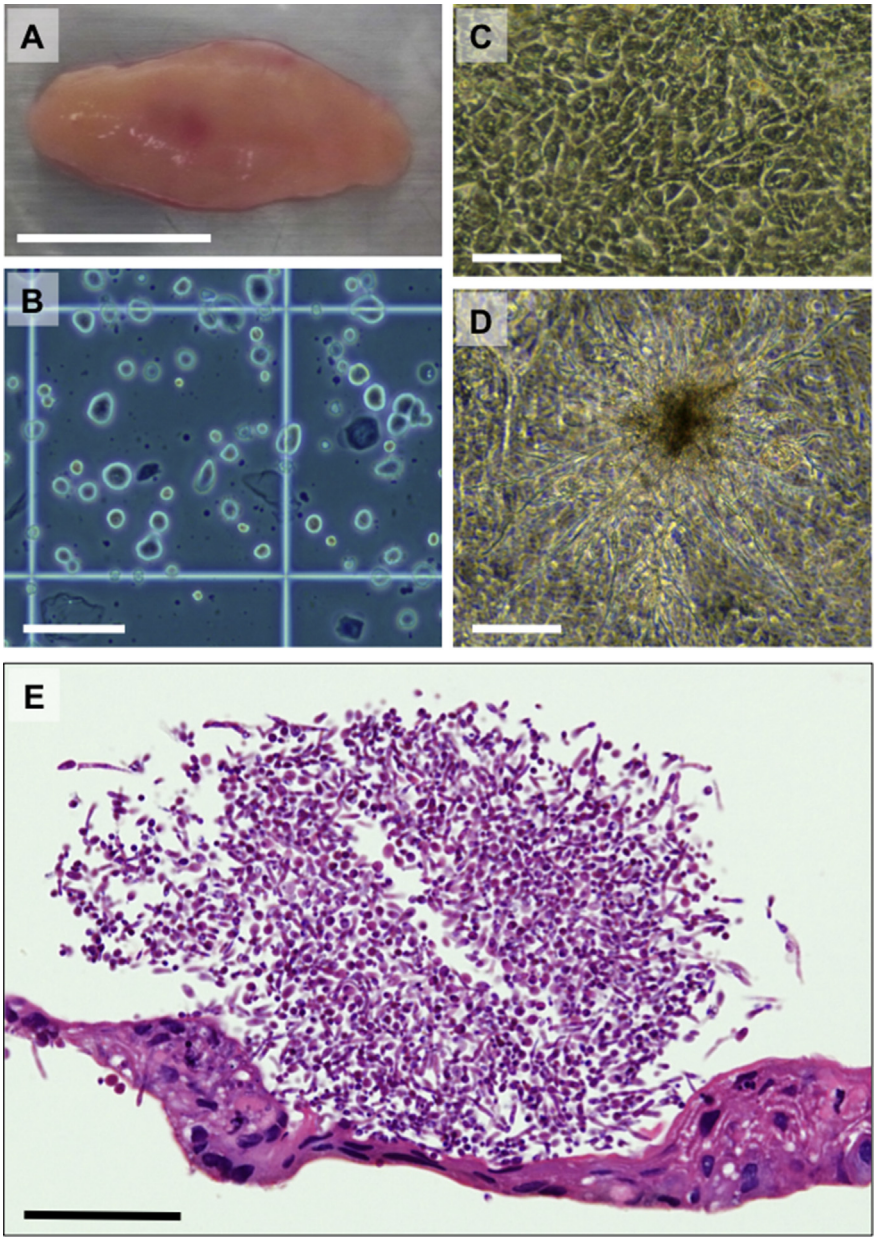


**Fig. 1.** *Candida albicans* (*C. albicans*) proliferating in the cell culture supernatant of human oral mucosal epithelial cells in this clinical study. (A) Human oral mucosal tissue of the patient. Bar = approximately 1 cm. (B) Oral mucosal epithelial cells derived from the patient after cell preparation. Bar = 100 μm. (C) Cellular morphology of the cultured human oral mucosal epithelial cells. Bar = 100 μm. (D) *C. albicans* observed on the cultured epithelial cells in a culture vessel. Bar = 100 μm. (E) Histological observation of the *C. albicans* adhering to a cultured epithelial cell sheet harvested from a temperature-responsive culture insert. The cell sheet and *C. albicans* were stained with hematoxylin and eosin. Bar = 50 μm.

patient, and the cultured epithelial cells were successfully harvested as cell sheets (Table 1, Fig. 2). To maintain a sterile environment, the temperature-responsive cell culture inserts to which the cultured epithelial cell sheets adhered were placed in transportable containers while in the safety cabinet of a clean room specialized for fabricating transplantable cell sheets for a clinical setting. The containers were then transported to Nagasaki University Hospital in the transportation box, which was mounted on a hot plate to keep the temperature at 37 °C. After transport, the epithelial cell sheets were finally transplanted onto the esophageal ulcer of the patient after endoscopic dissection to remove esophageal cancer.

Here, we have reported our experience of contamination with *C. albicans* during the fabrication of transplantable oral mucosal epithelial cell sheets derived from a patient who was not suffering from candidiasis. By adding 1 μg/mL amphotericin B to the transportation medium, fungal proliferation was completely inhibited and esophageal mucosal regeneration was successfully observed. Therefore, the method described in this report should be useful for preventing contamination with *C. albicans* without increasing the concentration of amphotericin B in the culture medium.

## Disclosure statement

Teruo Okano is a founder and director of the board of CellSeed Inc., licensing technologies and patents from Tokyo Women's Medical University. Teruo Okano and Masayuki Yamato are

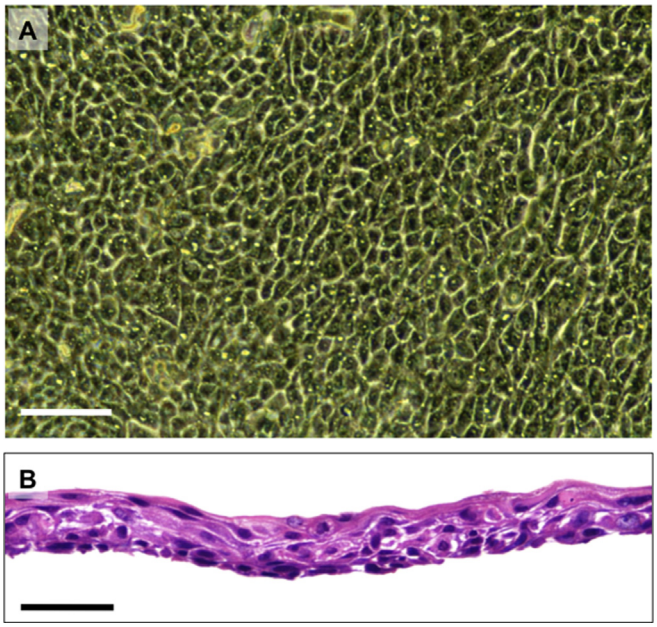


**Fig. 2.** Second trial of cultivation of human oral mucosal epithelial cells derived from the same patient, without contamination with bacteria or fungi. (A) Cellular morphology of the cultured human oral mucosal epithelial cells derived from the patient. Bar = 100 μm. (B) Histological observation of a cultured human oral mucosal epithelial cell sheet harvested from a temperature-responsive culture insert. The cell sheet was stained with hematoxylin and eosin. Bar = 50 μm.

shareholders of CellSeed Inc. Tokyo Women's Medical University is receiving research funding from CellSeed Inc.

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