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Yeasts from skin colonization are able to cross the acellular dermal matrix

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ABSTRACT

In recent decades, the prognosis for burn patients has improved considerably with the development of specialized care. The acellular dermal matrix (ADM) is a totally artificial acellular device that functions to control water loss, prevent penetration by bacteria and allow migration of endothelial cells and fibroblasts from patient tissues. However, little is known about its effectiveness against yeasts. The present study evaluated the capacity of colonization and migration of some human commensal yeasts. Three clinical isolates from skin scales, identified as *Candida parapsilosis*, *Candida glabrata* and *Rhodotorula mucilaginosa*, were used. Their ability to cross the ADM was evaluated. After three days, all isolates had crossed the ADM. *C. parapsilosis* showed the lowest growth, while *R. mucilaginosa* showed intermediate and *C. glabrata* the highest growth. In the plates incubated for seven days, the growth of *C. parapsilosis* and *C. glabrata* increased by 1 log over the third day. All isolates have the capacity to colonize and migrate through the matrix, increasing the potential risk to burn patients, who can develop severe and even fatal infections by invasive fungi.

1. Introduction

According to the World Health Association, burns are a global public-health problem. In the last decade, the prognosis for burned patients have improved substantially with the development of curative care, early debridement and broad-spectrum antibiotics; however, burns continue to cause considerable morbidity and mortality, and infections are among the main complications [1,2]. Reports indicate that 50–75% of the morbidity in patients with burns is related to infections, which have been a constant threat to human health throughout history, because they delay wound healing and can lead to death [3]. The most common pathogenic agents that cause serious infections in burn patients include *Staphylococcus aureus* or *Pseudomonas aeruginosa* [3–6]. Fungal infections have been increasing steadily in recent decades, and members of the genus *Candida* have been the main agents of these infections in burn patients, because they have impaired skin-barrier protection, which is susceptible [7].

Burn patients with impaired skin-barrier protection are susceptible to severe *Candida* infections [8]. Norbury et al. (2016) observed that fungi such as *Candida* non-*C. albicans* (CNCA) have emerged as important causes of invasive infections in burn patients, alongside antibiotic-resistant bacteria. However, little is known about the physiopathology of these infections in burn patients [9]. Yeasts of the genus *Rhodotorula* are saprophytes, able to colonize the human epithelium, respiratory tract and gastrointestinal tract. But, they are opportunistic pathogens from environmental sources or even from the patient's own microbiota, especially in immunocompromised patients, for example those who have suffered severe burns. *Rhodotorula* spp. is the third most common yeast isolated from blood cultures, and the species *R. mucilaginosa* is most often involved [10,11].

The initial procedure for the recovery of the burned area is aseptic debridement of the burn site. A dermal matrix is then implanted, and after the dermal matrix heals, a skin graft is performed [12]. The dermal regeneration matrix is a completely artificial acellular device, composed of two layers: the top layer is a thin silicone sheet, similar to the epidermis in its function to control water loss and prevent bacterial invasion. The pore size of the bottom layer allows endothelial cells and fibroblasts to migrate from the patient into the matrix. The anatomical structure and chemical composition of the artificial dermis provide a template for the synthesis of a structure similar to the patient's own skin [3,13]. However, this process may be impaired due to the growth of opportunistic microorganisms, and *in vitro* studies are important to evaluate this interaction between the device and the microorganisms. Only a few models have been developed to study the infection dynamics

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of a material used for treatment of burn patients [3,6].

The present study aimed to develop a model able to show the interaction between yeasts and a dermal matrix. We evaluated the capacity of colonization and migration of *Candida parapsilosis*, *Candida glabrata* and *Rhodotorula mucilaginosa* into this device, which is used in the restoration of the dermis of burn patients.

2. Materials and methods

2.1. Clinical isolates

Isolates of *Candida parapsilosis, Candida glabrata* and *Rhodotorula mucilaginosa* collected from skin scales were used. These yeasts were isolated from patients, identified by classical and molecular methods, and were deposited at Microbial Collections of Paraná Network- TAX online at Federal University of Paraná and on collection (Micoteca) of the Medical Mycology Laboratory, Laboratório de Ensino e Pesquisa em Análises Clínicas (LEPAC, UEM), with the identification codes: *C. parapsilosis* (CMRP2982), *C. glabrata* (CMRP2983) and *R. mucilaginosa* (CMRP2984). On LEPAC, Tthe yeasts were stored in Sabouraud Glucose Broth (SGB; Difco™, USA) with glycerol at −80 °C. Before the experiments, these isolates were subcultured in Sabouraud Glucose Agar (SGA; Difco, USA) and CHROMagar™ *Candida* (Difco, USA), to check the culture purity. After the yeasts were reactivated, their identities were confirmed with classical proofs of identification, i.e., observation of morphology and biochemical tests [14].

2.2. Acellular dermal matrix

The acellular dermal matrix (ADM) (PELNAC® artificial dermis, fenestrated type, size 120 × 240 mm; Gunze Ltd., Kyoto, Japan) is a material used in the restoration of dermis in patients who have suffered severe burns. The matrix has two sides, one hydrophobic (front) with a thin silicone layer, and the other hydrophilic (verse). To prepare the ADM for assay, squares of 1 × 1 cm^2 were cut with a scalpel aseptically, and the pieces of the array were hydrated for 15 min with sterile saline (0.9% NaCl) at room temperature, according to the manufacturer's instructions.

2.3. Inoculation of yeasts on ADM

In 24-well culture microplates (TPP, Switzerland) containing 5 ml of SGA with chloramphenicol, the pieces of hydrated ADM were deposited aseptically, with the verse in contact with the agar. On the center (front) of the ADM, 20 µl of a suspension containing 1 × 10^7/ml of *C. parapsilosis*, *C. glabrata* or *R. mucilaginosa* in sterile saline was added. Tests were performed in triplicate, on two independent days. A positive control (inoculum and SGA) and negative control (only ADM and SGA) were included. The plates were incubated at 35 °C for three and seven days. Times of incubation were chosen according to Fahrenbach et al. (2013) and inoculum size according Norbury et al. (2016).

2.4. Evaluation of yeast presence on the ADM

The presence of the yeast on the ADM after seven days of incubation was assessed with microscopy techniques (digital, epifluorescence and scanning electron microscopy, SEM). For digital microscopy (Color CMOS Sensor, High-Speed DSP) the pieces of ADM were carefully removed from the 24-well plate, fixed in 100% methanol for 15 min, and the front and verse surfaces photographed with a digital microscope at 800 × magnification. The epifluorescence microscopy (EVOS™ FL, Life Technologies) was performed with ADM stained with Calcofluor White (Fluka Analytical, Canada) diluted in a proportion of 1:4 in sterile phosphate-buffered saline 0.01 mol/L, pH 7.4 solution (PBS) for 5 min, and excess dye was removed by washing once with PBS. The stained ADM pieces were observed under a microscope with a filter capable of detecting the yeast cell wall (BP 365–370, FT 400, LP 421). For the SEM analysis, the ADM pieces were removed from the SGA plates and fixed with glutaraldehyde (2.5%) for 2 h. Then, the ADM pieces were dehydrated in a series of ethanol washes (70, 80, 90, 95, and 100%). The pieces were fixed on supports, coated with gold-palladium in argon atmosphere, using a gold-sputter module in a high-vacuum evaporator. The samples were observed with a Shimadzu SS-550 Superscan (Shimadzu, Tokyo, Japan) at 100, 1000 and 3000 × magnifications.

2.5. Evaluation of yeast ability to cross the ADM

The ability of the yeasts to cross the ADM was analyzed based on colony-forming units (CFU). After the incubation period (three and seven days), the ADM pieces containing the different strains were removed from each well. The surfaces of the SGA plates (which were in contact with the ADM) were scraped, and the remaining colonies were placed in tubes with 1 ml PBS and mixed vigorously on a vortex for 1 min. Then, suitable dilutions of the samples were inoculated onto SGA plates, followed by incubation at 37 °C for 48 h. The quantity of viable yeast was calculated by enumerating the CFUs.

2.6. Statistical analysis

Data with a non-normal distribution were expressed as the mean ± standard deviation (SD) of at least three independent experiments. Significant differences among means were identified using the Kruskal-Wallis test followed by Dunn's multiple-comparison test. The data were analyzed using Prism 6.0 software (GraphPad, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Yeast characterization

The yeasts were identified by classical methodology, and were confirmed as *C. parapsilosis*, *C. glabrata* and *R. mucilaginosa*.

3.2. The presence of yeast on the ADM

All isolates were capable of colonizing the ADM, as shown by digital microscopy (Fig. 1 A, G, M). Within seven days, optical (Fig. 1) and electron microscopy (Fig. 2) showed the formation of a complex structure formed by the yeasts on both sides (front and verse) of the ADM. As seen in Fig. 1, *C. parapsilosis* produced filaments on the hydrophobic (front) side, which were especially evident with epifluorescence microscopy (Fig. 1 B, C). Large increases in growth were seen in the *C. glabrata* and *R. mucilaginosa* samples and yeasts, without forming filamentous structures (Fig. 1 H, I, N, O). On the bottom of the matrix (Fig. 1 E, F, K, L, Q, R), there was an overgrowth of yeast.

Electron microscopy showed the growth and architecture formed by the yeast in more detail (Fig. 2), especially the interaction between the microorganisms and the device, on the front and verse of the ADM. For *C. parapsilosis,* filament-like structures were visible on both sides of the device (Fig. 2A–D). *C. glabrata* and *R. mucilaginosa* showed extensive growth (Fig. 2E–L), and all isolates were potentially able to colonize the ADM. The negative control was tested to demonstrate the structure and sterility of the ADM (Fig. 2M–P). Fig. 3 shows that a drop containing the inoculum deposited under the ADM did not overflow the edges of the device, remaining where it was deposited during the entire culture period. Taken together, these observations indicate that all the yeasts migrated through the ADM.

3.3. Ability of yeasts to cross the ADM

Within three days, the isolates of *C. parapsilosis*, *C. glabrata* and *R. mucilaginosa* were able to cross the ADM and reach the agar. *C.*