Utilization of an in vitro biofabricated 3D skin as a pathological model of cutaneous candidiasis

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SUMMARY

*Candida albicans* is an opportunistic fungal infectious agent that can cause cutaneous candidiasis in humans. Biofilms formation of *C. albicans* is thought to be the major cause of antifungal drug resistance. Despite numerous studies conducted on *C. albicans* biofilms, a comprehensive understanding of how *C. albicans* biofilms induced cutaneous candidiasis in humans and the development of a more effective targeted therapy remain poorly investigated. Available animal models of cutaneous candidiasis and in vitro human skin cell cultures do not fully reflect the actual human skin microenvironment or the disease pathogenesis. We investigated the molecular pathology of *C. albicans* infection using an in vitro biofabricated 3D skin. This in vitro biofabricated 3D skin comprises a fully humanized three-dimensional (3D) skin equivalent, consisting of a stratified terminally differentiated epidermis and an underlying dermal compartment. Antifungal drug susceptibility testing, histological and electron microscopy study, biofilms study, and pro-inflammatory cytokines analysis were conducted in *C. albicans* infected skin. Histological results revealed that *C. albicans* covered and produced biofilm on the biofabricated 3D skin, invading the skin compartments including epidermis and dermis. Elevation of proinflammatory cytokines including MMP-9, IL-1β, TNF-α, and IL-5 were examined in the *C. albicans* infected skin. However, treatment with itraconazole reduced the pathology of *C. albicans* infection. This study provides an alternative pathological model of cutaneous candidiasis, which can physiologically represent a close-up event during *C. albicans*. Moreover, it is rapid, cost-effective, and reproducible of the in vitro biofabricated 3D skin model, and may further highlight the importance of utilizing in vivo-like conditions to improve high-throughput screening for drug discovery against several antifungal drug-resistant pathogens.

INTRODUCTION

Cutaneous infections with the opportunistic pathogenic yeast *Candida albicans* are commonly found throughout the world (Taudorf et al., 2019). Variations in the cutaneous environment, antibiotic treatment, underlying diseases, and alterations of skin immunity can trigger the switch of *C. albicans* from the commensal form into the pathogenic form, resulting in significant infectious processes (Gow et al., 2011). Common pathological sites of infection are warm, moist, and creased areas such as the genitals, inflammatory folds, and/or intestinal mucosa in up to 70% of healthy individuals (Perlroth et al., 2007). Interestingly, appearances of cutaneous candidiasis can be used as a clinical marker for innate or acquired immune deficiency/dysfunction. Patients with HIV infection, diabetes mellitus, chemotherapy-induced neutropenia or the use of chronic systemic corticosteroid are susceptible to *Candida* skin infection, leading to cutaneous candidiasis (Cassone and Cauda, 2012; Rodrigues et al., 2019; Fardet et al., 2016). Numerous studies have highlighted the virulence factors that are expressed or required by *C. albicans* to cause skin infection, with hypha, cell-wall activation, and biofilm formations playing a major role in the infectious process (Mayer et al., 2013). Although the mechanisms of biofilm resistance to antifungal agents are not fully elucidated, various in vitro models have been established to investigate the properties of *C. albicans* biofilms as well as to test the activity of new potential antifungal drugs against *C. albicans* (Douglas, 2003). Data from several studies have demonstrated that *Candida* biofilms are highly resistant to clinically approved antifungal drugs including amphotericin B, fluconazole, itraconazole, ketoconazole, and new azoles such as voriconazole (Baillie and Douglas, 1998; Chandra et al., 2011; Hawser and Douglas, 1995; Lewis et al., 2002). Traditional drug discovery is often based on 2D monolayer culture conditions; however, 2D cell monolayers in vitro fail to reproduce the interconnections between target cells to their extracellular matrix (ECM) environment (Langhans, 2018). As a result, results from in vitro models of *Candida* biofilms may not fully represent biofilm structures and mechanism of antifungal agents both qualitatively and quantitatively, and thus cannot be directly applied to patients with candidiasis.

Key words: *In vitro* biofabricated 3D skin, *Candida albicans*, Cutaneous candidiasis, Biofilms, Matrix metalloproteinase-9 (MMP-9), Interleukin-1β (IL-1β), Tumor necrosis factor-α (TNF-α), Interleukin-5 (IL-5).

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The wide ranges of susceptibility to *C. albicans* infection have suggested the existence of tailored immune responses localized at skin compartments. Advanced studies with genetic mouse models have gained insights into the mechanisms of cutaneous candidiasis in response to innate and adaptive immunity (Kashem and Kaplan, 2016). A previous study in an experimental model of keratomycosis has demonstrated that the inflammation caused by *C. albicans* infection can lead to secretion of a significant amount of matrix metalloproteinase (MMP)-9, which further destroys epithelial cell-base membrane proteins and promotes *C. albicans* invasion (Yuan et al., 2009). Moreover, elevation of interleukin (IL)-5 secretion during Malassezia globosa infection has been shown to be involved with production of tumor necrosis factor (TNF)-α leading to cutaneous inflammation, immune response, and allergic conditions (Ishibashi et al., 2006). Collectively, these data suggest that MMP-9, IL-5, and TNF-α may represent a host-defense against *C. albicans* infection; however, the biological mechanisms underlying host-*Candida* interactions in localized human skin compartments is still not fully elucidated.

Direct study of drug discovery and the interaction between fungi and human epithelial surface like skin are still limited. In particular, interactions of these two organisms are complex and involve several stromal cells underneath the epithelium in a tissue specific manner. Several animal models of fungal infection have been established; however, these models differ substantially from the human host, resulting in inconsistent results and ethical considerations (Barré-Sinoussi and Montagutelli, 2015). Due to the recent technological advancements, three-dimensional (3D) cell culture is gaining momentum, providing a better, fast, cost-effective, and easily reproducible platform for drug testing (Ryan et al., 2016).

To elucidate more accurately the pathogenesis of cutaneous candidiasis caused by *C. albicans*, we established a cutaneous infection model using an *in vitro* biofabricated 3D skin. Using this model, we investigated the effects of antifungal drugs on *C. albicans* biofilm formation and the role of MMP-9 and other related inflammatory cytokines. Our study provides an advantage by utilizing an *in vitro* biofabricated 3D skin, which can aid further study of cutaneous pathogenesis and drug discovery with cost-effectiveness in pre-clinical testing and advance innovation in personalized medicine.

**MATERIALS AND METHODS**

*Candida albicans* strains

Two strains of *C. albicans* were obtained from American Type Culture Collection (ATCC) including: *C. albicans* SC-5314, a biofilm producing strain, and *C. albicans* 90028, an anti-fungal susceptibility testing strain. Both *C. albicans* strains were propagated overnight on Sabouraud Dextrose Agar (SDA) (Oxoid, Hampshire, UK) slant at 37°C. Then, cells were cultured overnight in Yeast Peptone Dextrose Broth (YPDB) (containing 2% peptone, 1% yeast extract, and 2% glucose) at 30°C to produce a culture of yeast cells. Yeast cells were collected and washed twice with phosphate-buffered saline (PBS, pH 7.2), counted with a hemocytometer, and adjusted to 5x10⁷ cells/ml in YPDB.

In vitro fungal growth kinetics

To determine the growth kinetics of *C. albicans*, human melanoma cell line (CRL-1676, ATCC) was used in this study. CRL-1676 cells were cultured in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin and maintained at 37°C with 5% CO₂ in a humidified incubator prior to analysis.

For fungal growth kinetics, CRL-1676 cells were cultured on a cover glass slip in six-well plates at a density of 1x10⁶ cells/well. After overnight attachment, they were stimulated by *C. albicans* SC-5314 or untreated controls. At 2, 6, and 12 h post-infection, supernatants were collected and stored at -20°C until used to measure cytokine levels and enzymatic activity. The cells were further examined for morphological changes using Hematoxylin & Eosin (H&E) staining and scanning electron microscopy (SEM). For SEM, infected cells were first fixed in 2.5% glutaraldehyde for 1 h at room temperature and washed 3 times with 0.1 M sucrose phosphate buffer (SPB). The slides were then fixed in 1% osmium tetroxide in SPB and washed again. The slides were dehydrated in a series of ethanol and air-dried overnight. The slides were mounted on an aluminum stub and coated with a gold film (20 nm-thickness) using a sputter coater (Emitech K550, Ashford, UK). The luminal surface of catheter samples was imaged using a scanning electron microscope (JEOL JSM-6610LV, Japan) with 15 kV acceleration voltages.

Anti-fungal susceptibility

To determine anti-fungal susceptibility, the minimum inhibitory concentrations (MICs) of *C. albicans* SC-5314 were determined according to recommendations stated in the Clinical and Laboratory Standards Institute (CLSI) M27-A3 document (CLSI, 2008). Anti-fungal drugs used in this study were obtained as reagent-grade powders for CLSI method including amphotericin B, fluconazole, itraconazole, voriconazole, and posaconazole (Sigma, St. Louis, MO, USA). Briefly, *C. albicans* inoculums were diluted in RPMI 1640 medium and the final inoculum in assay wells was between 0.5x10⁻⁵-5x10⁻⁷ CFU/ml. The *C. albicans* inoculums were incubated at 35°C for 24-48h. In the CLSI method, the MICs were determined visually after 24 h of incubation, which selected the lowest concentration of a drug that caused ≥50% inhibition in growth compared to drug-free controls. The quality control was determined using *C. albicans* 90028.

Biofabricated three-dimensional (3D) skin model

Experiments were performed using viable biofabricated three-dimensional (3D) human skin equivalent containing co-cultured normal human-derived epidermal keratinocytes (NHK) and melanocytes (NHM) obtained from Asian donors (MatTek Corporation, Ashland, MA, USA). The NHK cells were seeded with NHM at a 10:1 ratio on a nylon mesh as basement membrane. The artificial biofabricated 3D skin exhibited melanogenesis and formed a multilayered cell culture model similar to human skin morphology (Aramwit et al., 2018). A ready-to-use artificial skin was maintained and fed every other day with fresh EPI-100-LLMM-4AFAB-specific complete medium without anti-fungals in 24-well plates without sub-culturing at 37°C with 5% CO₂ in a humidified incubator. The artificial tissues were washed with PBS to remove any residual test compound prior to analysis.
In vitro cutaneous candidiasis model

Artificial biofabricated 3D skin were used as an in vitro model to examine the pathology of cutaneous candidiasis in human equivalent skin. Briefly, artificial skins were co-cultured with 100 μl of C. albicans SC-5314 (10⁶ cells/ml in RPMI 1640) for 24 h, and then treated with 100 μl of itraconazole (0.75 μg/ml) for 6 h. Untreated artificial skins co-cultured with C. albicans SC-5314 were used as controls. At 6 h post-treatment, artificial skins were divided into two sections, either:

1) fixed in 10% neutral buffer formalin for histopathological studies or
2) fixed with 2.5% (v/v) glutaraldehyde in sucrose phosphate buffer (SPB) for transmission electron microscopy (TEM).

Assessment of the C. albicans biofilm development

H-Score analysis: Upon treatment of itraconazole on artificial biofabricated 3D skins co-cultured with C. albicans SC-5314, the semi-quantitative examination of the C. albicans biofilm formation was measured by the H-score assay. Similar to an in vitro cutaneous candidiasis model, C. albicans SC-5314 infected artificial skins were treated with 0.75 μg/ml of itraconazole. Untreated C. albicans SC-5314 infected artificial skins were used as controls. Then, 10 fields of biofilms were randomly selected and photographically captured at 1,000x magnification at 2, 4, 6, 8, 24, and 48 h post-treatment. The H-score index was applied by multiplying the percentages of covered area of biofilm with the layer score of biofilm thickness (1 = one layer; 2 = two layers, and 3 = three or more layers of biofilm thickness) using ImageJ program (National Institutes of Health, Bethesda, MD, USA).

Crystal violet (CV) staining assay: The C. albicans biofilm formation in an in vitro cutaneous candidiasis model was further quantified using crystal violet (CV) staining as previously described by Pumeesat et al., 2017. After itraconazole (0.75 μg/ml) treatment of C. albicans SC-5314 infected artificial skins were washed twice with 200 μl of sterile PBS and allowed to air-dry for 45 min at 2, 4, 6, 8, 24, and 48 h post-treatment. Then, pre-washed wells were stained with 110 μl of 0.4% aqueous CV solution for 45 min. Stained wells were washed five times with 350 μl sterile distilled water; and then destained with 200 μl of 95% acetic acid solution. Stained wells were scanned by a gel documentation system (Bio-Rad Laboratories, UK) to detect active MMPs activity.

Western blot: To determine the matrix metalloproteinase-9 (MMP-9) proteins in artificial biofabricated 3D skins co-cultured with C. albicans SC-5314, western blotting was performed as previously described (Suwanmanee et al., 2019). The infected artificial skins culture supernatant containing MMP-9 proteins was lysed to obtain the total protein by radioimmuno-precipitation assay at 24 h post-treatment of 0.75 μg/ml itraconazole. Over-stimulation of MMPs by 100 ng/ml of phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MO) treatment in non-infected artificial skins was used as a positive control (Shin et al., 2007). The gel was scanned by a gel documentation system (Bio-Rad Laboratories, UK) to detect active MMPs activity.

Pathogenesis study of cutaneous candidiasis

Detection of matrix metalloproteinases (MMPs) activity: The activity of active MMPs in C. albicans SC-5314 infected artificial skins was investigated by using sodium dodecyl sulfate polyacrylamide (SDS-PAGE) zymography using a gelatin substrate (Luplertlop and Missé, 2008). Briefly, 500 μl aliquot of infected artificial skins culture supernatant was collected at 24 h post-treatment of 0.75 μg/ml itraconazole. The sample supernatants were lyophilized and dissolved in loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 0.04% bromophenol blue) and electrophoresed on a 7.5% SDS-polyacrylamide gel containing 1 mg/ml gelatin. Then the gel was stained with Coomassie brilliant blue R-250 and destained in 30% (v/v) methanol/10% (v/v) acetic acid solution. Over-stimulation of MMPs by 100 ng/ml of phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MO) treatment in non-infected artificial skins was used as a positive control (Shin et al., 2007). The gel was scanned by a gel documentation system (Bio-Rad Laboratories, UK) to detect active MMPs activity.

Fluorescent bead-based measurement of cytokines analysis: Cytokine measurements (MMP-9, IL-1β, TNF-α, and IL-5) were performed with 20 μl supernatants (from infected artificial skins at 2 h and 24 h post-infection) using the Bio-Plex Pro Human Cytokine 8-Plex Immunoassay according to manufacturer instructions. Uninfected artificial skin supernatants were used as control. The standard curves were

and incubated in the dark for 2 h at 37°C. Next, 100 μl of supernatant were transferred to a new microtiter plate and absorbance values were measured with a microtiter plate reader at 490 nm using a microtiter plate reader. Data was calculated as arithmetic mean of the absorbance values. The absorbance values of negative control wells (mock infected controls) were subtracted from the tested wells to eliminate any absorbance background.
automatically optimized by the Bioplex manager software and verified manually. The Bioplex manager software was used to calculate cytokine concentrations and only measurements that showed a coefficient of variability (CV) of <10% were included for analysis.

**Statistical analysis:** Each experiment was performed in triplicate. The data was presented as the mean ± standard deviation (SD). One-way ANOVA or independent Student's t-tests were used to assess differences between groups. Significant differences were considered at the p-values <0.05.

**RESULTS**

*In vitro fungal growth kinetics*

A human melanoma cell line (CRL-1676) was inoculated with a biofilm producing strain of *C. albicans* (SC-5314) to determine a fungal growth kinetic of *C. albicans* SC-5314 at 2, 6, and 12 h post-inoculation (Figure 1). The hypha formation was already seen at 2 h post-inoculation (Figure 1A). Interestingly, the results revealed that *C. albicans* was fully grown on CRL-1676 monolayer at 12 h post-infection (Figure 1C). Scanning electron micrograph of *C. albicans* infected CRL-1676 at 12 h post-inoculation showed a prominent presence of several pseudohyphaes in both short and elongated forms as true hyphae rather than budding yeast forms (Figure 1D).

**Determination of Minimum Inhibitory Concentration (MIC)**

The ranges of Minimum Inhibitory Concentration (MIC) and the MIC<sub>50</sub> and MIC<sub>90</sub> values of the tested anti-fungal drugs for a biofilm producing strain (SC-5314) and an anti-fungal susceptibility testing strain (90028) of *C. albicans* are presented in Table 1. In the present study, most of the anti-fungal drugs showed MIC range of 0.016-264 μg/ml. Moreover, itraconazole, voriconazole, and posaconazole were notably more active in inhibiting the growth of *C. albicans* SC-5314 and 90028 than were amphotericin B and fluconazole. In addition, itraconazole is commonly known as a standard treatment for Candidiasis (Del Rosso et al., 1998). Thus, itraconazole at the concentration of 0.75 μg/ml was selected for further experiments in this study (Table 1).

*Cutaneous candidiasis of artificial biofabricated 3D skin*

Histopathological features of *C. albicans* SC-5314 inoculated artificial biofabricated 3D skin were investigated at 6 h post-treatment with 0.75 μg/ml of itraconazole, and compared to untreated controls (Figure 2A-D). The results revealed that *C. albicans* were fully grown in untreated skin (Figure 2A). The untreated skin section also showed the invasion of *C. albicans* especially in the upper surface of the artificial biofabricated 3D skin, which is equivalent to the stratum corneum of human skin (Figure 2B). Transmitting electron micrograph of untreated skin also

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**Figure 1** - Growth of *C. albicans* SC-5314 on human melanoma cell line (CRL-1676). Histopathological appearance of CRL-1676 monolayer was determined after inoculation with *C. albicans* at (A) 2 h, (B) 6 h, and (C) 12 h post-inoculation. (D) CRL-1676 monolayer after infection with *C. albicans* at 12 h post-inoculation was further observed under a scanning electron microscope (SEM). Scale bar =20 and 5 μm.
demonstrated the invasion of *C. albicans* into the upper layer of the skin (Figure 2E, F). This data showed that *C. albicans* is able to propagate on the artificial biofabricated 3D skin. However, treatment with 0.75 μg/ml of itraconazole significantly reduced the growth of *C. albicans* (Figure 2C). In the upper surface of the itraconazole-treated skin, less hyphal-like structures were detectable compared to the untreated controls (Figure 2D).

**Assessment of the *C. albicans* biofilm development**

**Quantification of biofilm by H-Score analysis: *C. albicans* SC-5314 biofilm development on the upper layer of untreated and itraconazole (0.75 μg/ml) treated artificial biofabricated 3D skin started at 2 h post-treatment. At 2 h post-treatment, *C. albicans* culture was visible as a single layer of long hyphae on the skin (thickness score =1). At 4 h post-treatment, the biofilms were formed as two layers (thickness score =2). At 6 h post-treatment (6-48 h), multiple layers of *C. albicans* biofilms were observed (thickness score =3). *C. albicans* biofilm was further determined using H-score analysis. The percent coverage area of biofilm multiplied with the layer score of the *C. albicans* SC-5314 biofilm was calculated and represented as H-scores. In the present study, the means of H-scores in *C. albicans* inoculation of untreated and itraconazole treated skins were gradually increased from 2 h to 6 h but declined at 6 h post-treatment (Figure 3A). The mean H-scores of *C. albicans* biofilm development of untreated skin was significantly higher than itraconazole treated skin (p<0.05) at 4 h (162.03±1.38 vs 134.96±3.64), 6 h (398.99±3.01 vs 148.00±4.01), 8 h (299.47±1.88 vs 195.16±6.38), 24 h (233.43±1.99 vs 104.06±4.14), and 48 h (175.16±2.35 vs 92.11±5.37) post-treatment. However, at the early period of treatment (at 2 h), the mean H-scores of the untreated skin was insignificant when compared to the treated skin (p>0.05, 29.35±1.44 vs 24.57±1.09).

**Quantification of biofilm by crystal violet (CV) assay:** Fungal biofilm on the upper layer of untreated and itraconazole (0.75 μg/ml) treated artificial biofabricated 3D skin was further determined at 2, 4, 6, 8, 24, and 48 h post-treatment using crystal violet staining. The biofilm of *C. albicans* SC-5314 in untreated and itraconazole treated skins gradually increased from 2 h to 6 h but declined at 6 h post-treatment (Figure 3B). Similar to H-score analysis, the pattern of *C. albicans* biofilm development was similar in untreated and itraconazole-treated skin (p<0.05) at 4 h (1.88±0.09 vs 0.38±0.17), 6 h (3.14±0.27 vs 1.97±0.22), 8 h (2.08±0.19 vs 1.13±0.68), 24 h (1.83±0.03 vs 0.79±0.08), and 48 h (1.19±0.18 vs 0.51±0.24) post-treatment. Notably, at the first time-point examined (2 h), the mean OD95% of the untreated skin was not significantly different compared to the treated skin (p>0.05, 0.39±0.16 vs 0.28±0.11).

**Quantification of biofilm by XTT assay:** Biofilm metabolic activity of *C. albicans* SC-5314 on the upper layer of untreated and itraconazole (0.75 μg/ml) treated artificial biofabricated 3D skin was further determined at 2, 4, 6, 8, 24, and 48 h post-treatment using XTT reduction assay. In this study, the results showed that biofilm metabolic activity of untreated skin was significantly higher than itraconazole treated skin at 4 to 6 h post-treatment (p<0.05, 0.29±0.02 vs 0.16±0.03 and 0.43±0.01 vs 0.30±0.03, respectively, Figure 3C).
Moreover, *C. albicans* biofilm metabolic activity was not significantly different (*p* > 0.05) between untreated and itraconazole treated skin at 2 h (0.03 ± 0.01 vs 0.02 ± 0.01), 8 h (0.21 ± 0.03 vs 0.20 ± 0.04), 24 h (0.19 ± 0.01 vs 0.17 ± 0.05), and 48 h (0.15 ± 0.02 vs 0.11 ± 0.01) post-treatment.

**Expression of matrix metalloproteinase-9 (MMP-9):** Artificial biofabricated 3D skin was inoculated with *C. albicans* SC-5314 and the metalloproteinase activity in culture supernatants from untreated and itraconazole (0.75 μg/ml) treated skin was analyzed by gelatin zymography at 24 h post-treatment (*Figure 4A*). Phorbol myristate acetate (100 ng/ml, PMA) was used to over-stimulate the MMPs in normal skin as a positive control. The results revealed that normal skin constitutively secreted a basal level of activated MMP-9 (~92 kDa gelatinase). MMP-9 activation was strongly seen after inoculation of *C. albicans* similar to PMA stimulation. However, the strong activation of MMP-9 was reduced after treatment with 0.75 μg/ml of itraconazole (*Figure 4A*).

The activation of MMP-9 in skin inoculated with *C. albicans* was further determined using western blotting at 24 h post-treatment (*Figure 4B*). Secretion of MMP-9 protein was over-stimulated after incubation with *C. albicans*, similarly to what observed for PMA stimulation (~82 kDa MMP-9) when compared to control. However, the secretion of MMP-9 was reduced after treatment with itraconazole (*Figure 4B*).

**Expression of pro-inflammatory cytokines:** The levels of MMP-9 and pro-inflammatory cytokines associated with skin inflammation in *C. albicans* inoculated artificial biofabricated 3D skin were determined at 2 h and 24 h post-treatment with itraconazole (0.75 μg/ml) using Bio-Plex Pro Human Cytokine 8-Plex Immunoassay. The results showed that levels of MMP-9 gradually increased from 2h to 24h post-treatment in untreated skin with *C. albicans*. However, treatment of itraconazole (0.75 μg/ml) significantly decreased the MMP-9 levels at 2 h post-treatment but not at 24h post-treatment (*p* < 0.05, *Figure 4C*). On the other hand, levels of interleukin-1 (IL-1)-β, tumor necrosis factor (TNF)-β, and interleukin-5 (IL-5) were significantly increased at 2 h to 24 h post-treatment. Treatment of itraconazole (0.75 μg/ml), however, significantly decreased the IL-1β, TNF-β, and IL-5 levels in a time-dependent manner (*p* > 0.05, *Figure 4D-F*).

**DISCUSSION**

The formation of biofilms is a major virulence factor for fungal pathogenicity and is often correlated with antifungal drug resistance (Costa-Orlandi et al., 2017). *Candida* biofilms are among the most widely studied and *C. albicans* is still the most frequently isolated species found in cutaneous candidiasis (Reyes-Montes et al., 2017). Data deriving from *in vivo* analysis of *Candida* biofilms conducted on physical and biological materials such as catheters, dentures, or mucosal surfaces of animals are often unsuitable to provide evidences for drug discovery for cutaneous candidiasis (Reyes-Montes et al., 2017). Misinterpretation of data and unnecessary sacrifice of experimental animals as well as cost-benefit concerns remain relevant issues to be considered in order to achieve the most effective and applicable results. Ethical problems and incomprehensive understanding of *in vivo* models often limit the translation of research data acquired from animal studies into human clinical practice (Barré-Sinoussi and Montagutelli, 2015).

Here we used an *in vitro* biofabricated 3D skin as a pathological model of cutaneous candidiasis. This *in vitro* human skin equivalent has been developed to overcome the above challenges in elucidating disease pathogenesis,
An in vitro biofabricated 3D skin model of cutaneous candidiasis thereby improving the success rate of pre-clinical drug screening (Randall et al., 2018). We previously described a full thickness of human skin equivalent. Histological studies revealed that humanized skin compartments include keratinocytes, melanocytes, and fibroblasts. Moreover, an in vitro biofabricated 3D skin has been shown to recreate several molecular features of human skin, including the production of human extracellular matrix components, basement membrane proteins, and others related proteins (Aramwit et al., 2018). Thus, in this study, we performed an experimental C. albicans infection on an in vitro biofabricated 3D skin.

Our results showed that C. albicans can grow and produce biofilms on human melanoma cells in vitro. In general, pathogenic cells embedded in their secreted biofilms are significantly less susceptible to antimicrobial agents (Borghi et al., 2016). Our SEM analysis revealed that C. albicans biofilms covered the surface of human melanoma cells. Scanning electron micrograph also revealed that C. albicans biofilm microenvironment consists of a mixture of keratinocytes, melanocytes, and fibroblasts.

Figure 4 - Expression of matrix metalloproteinase-9 (MMP-9), interleukin-1 (IL-1)-β, tumor necrosis factor (TNF)-α, and interleukin-5 (IL-5) after C. albicans SC-5314 inoculation on untreated and itraconazole (0.75 μg/ml) treated artificial biofabricated 3D skin. The MMP-9 expressions were determined by (A) gelatin zymography, (B) western blotting analysis at 24 h post-treatment. Phorbol myristate acetate (100 ng/ml, PMA) was used to over-stimulate the MMPs as a positive control. The cytokine measurements of (C) MMP-9, (D) IL-1β, (E) TNF-α, and (F) IL-5 in the skin were determined using Bio-Plex Pro Human Cytokine 8-Plex Immunoassay at 2 h and 24 h post-treatment. Results are presented as mean ± standard deviation (SD). *indicated as significant difference at p < 0.05 by 2-way ANOVA.
of yeasts, hyphae, and pseudohyphae surrounded by extracellular matrix materials, which contributed to antifungal drug resistance (Tsui et al., 2016).

We also performed antifungal drug susceptibility testing against C. albicans biofilms. The results showed that itraconazole (0.75 μg/ml) effectively inhibited C. albicans biofilms formation in vitro. The MIC of all the tested antifungal drugs were similar to what previously reported (e.g., 0.016-2 μg/ml for amphotericin B; 0.016-64 μg/ml for fluconazole and itraconazole; and 0.016-16 μg/ml for voriconazole and posaconazole (Baghdadi et al., 2016)), suggesting the susceptibility of our C. albicans isolates to acceptable antifungal ranges. The antifungal effect of itraconazole (0.75 μg/ml) was further examined in C. albicans biofilms on in vitro biofabricated 3D skin. Histological results from untreated skin revealed that C. albicans biofilms covered the surface of human skin equivalent in a time dependent manner. Transmitting electron micrograph also revealed C. albicans conidia inside the epithelial cells of the skin equivalent, suggesting fungal invasion and propagation. Moreover, treatment with itraconazole (0.75 μg/ml) was effective in reducing the formation of C. albicans biofilms. Treatment with itraconazole (0.75 μg/ml) significantly reduced H-scores when compared to untreated control in a time dependent manner, suggesting that itraconazole is effective in controlling C. albicans biofilm formation. In addition, we measured the cellular community in C. albicans biofilms by CV staining (Melo et al., 2011), showing that the cellular biofilm community on the surface of in vitro biofabricated 3D skin was reduced after treatment with itraconazole. These results were consistent with XTT activity. Cell viability in biofilms can be measured using XTT, which reduces to formazan by mitochondrial dehydrogenase in lived fungal cells (Jin et al., 2003; Melo et al., 2011). In the present study, treatment with itraconazole significantly reduced the C. albicans biofilms growing on the infected in vitro biofabricated 3D skin, which showed as lower XTT metabolic activity than in untreated skin. Thus, our study demonstrated that an in vitro biofabricated 3D skin could be used as a pathological model to study the C. albicans biofilms formation in vitro. The expression of metalloproteinases (MMPs) has been previously investigated in a mouse model of keratomycosis induced by C. albicans strain SC5314 (Yuan et al., 2009). In this study, up-regulation of MMP-9 mRNA expression in the epithelium tissue was monitored during the progression of keratitis. Their findings suggested that expression of MMP-9 promotes the invasion of C. albicans into keratinocytes. Previous study has demonstrated that elevation of IL-5 production from human keratinocytes during M. globosa infection played an important role in cutaneous inflammation, immune responses, and allergic reactions (Ishibashi et al., 2006). Moreover, secretion of IL-1β, TNF-α, and MMPs can also be produced from human keratinocytes to synergistically trigger or exacerbate the immune response against M. globosa infection. Another study demonstrated that mRNA expression of TNF-α was also reduced in mice deficient for IL-17A (IL-17A−/−), suggesting the dependent regulation of TNF-α and IL-17A during anti-inflammatory response following C. albicans infection. Recently, a study on oral candidiasis in mice revealed that mice deficient with IL-1 (IL-1R1−/−) displayed down-regulation of IL-17 and IL-17-dependent genes (Verma et al., 2018), one of its targets being MMP-9 (Mays et al., 2006; Koslawsy et al., 2018). In the present study, we found that infection of C. albicans biofilms in an in vitro biofabricated 3D skin significantly increased MMP-9 enzymatic activity by gelatin zymography and western blotting (Figure 4A, B). At 2 h post-treatment, levels of MMP-9, IL-1β, TNF-α, and IL-5 pro-inflammatory cytokines were increased in untreated C. albicans-infected skin, but remarkably reduced in presence of itraconazole (Figure 4C-F). IL-1β, TNF-α, IL-5, but not MMP-9 levels were significantly reduced after treatment of itraconazole in a time dependent manner (24 h post-treatment). Elevation of MMP-9 after C. albicans infection of epithelia signals the extravasation of leukocytes and chemotaxis (Rohini et al., 2007). Moreover, MMP-9 is elevated soon after microbial inoculation, suggesting a key role for MMP-9 during injury and inflammation (Yuan et al., 2009). MMP-9 is also released by keratinocytes to potentiate superficial wound healing after fungal infection (Carter et al., 2007). Therefore, elevation of MMP-9 at 24 h post-itraconazole treatment may be due, at least in part, to the promotion of necrotizing inflammation and tissue repair by MMP-9. Further studies are necessary to elucidate the molecular mechanisms of MMP-9 and other related pro-inflammato-ry cytokines in the artificial biofabricated 3D skins during C. albicans infection. Collectively, our results demonstrate an alternative model of C. albicans infection in an in vitro biofabricated 3D skin, which exhibit activities and expressions of several pro-inflammatory profiles previously found in mice models. Moreover, treatment with itraconazole remarkably reduces biofilms formation and inflammation caused by C. albicans.

From the clinical point of view, the data presented in this study support the use of using an in vitro biofabricated 3D skin to investigate the pathogenesis of cutaneous candidiasis. Moreover, our results suggest that reduced MMP-9, IL-1β, TNF-α, and IL-5 production may help prevent C. albicans cutaneous infections in human. Interestingly, application of an in vitro biofabricated 3D skin might be beneficial for rapid anti-fungal drug screening in pre-clinical study, which plays an important role in the further development of immunomodulatory therapy, promising plant and animal extracts for antifungal activity, or vaccine development against C. albicans and other pathogenic infections.

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References


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An in vitro biofabricated 3D skin model of cutaneous candidiasis


