Biochem. Cell. Arch. Vol. 21, No. 2, pp. 3267-3272, 2021	www.connectjournals.com/bca	*ISSN 0972-5075*
DocID: https://connectjournals.com/03896.2021.21.3267		*eISSN 0976-1772*

BIOFILM AND EPITHELIAL THICKNESS IN TONGUE OF WISTAR RATS (*RATTUS NOVERGICUS*) INDUCED BY *CANDIDA ALBICANS* WITH IMMUNOSUPPRESSION CONDITION

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(Received 11 April 2021, Revised 6 June 2021, Accepted 19 June 2021)

ABSTRACT : Immunosuppression can occur due to the long-term use of corticosteroids making the host is susceptible to infection. *Candida* existed, especially *Candida albicans* as the main agent, in the oral cavity can change if the host is weaker and becomes an opportunistic pathogen. They will create a colonization which is called biofilm which is able to penetrate and damage the epithelium. This condition leads to the oral candidiasis and the thinning of the epithelium. This research aimed to investigate biofilm and epithelial thickness on the tounge of rats induced by *Candida albicans* ATCC-10231 with immunosuppression conditions. This study was a true experimental study with a posttest-only control group design. Fifteen healthy male Wistar Rats (*Rattus novergicus*), aged around 12 weeks old and weighted around 200-250 g were immunosuppressed through oral administration of dexamethasone and tetracycline for 21 days, then induced with *C. albicans* (ATCC-10231) 1-Mc.Farland. The subjects were divided into three groups (n=5/group); healthy (HG), Immunosupressed+*C. albicans* (ICAG), and nystatin treatment (NTG). The subjects were treated for 14 days, later the rats were euthanized, and their tongues were biopsied. Biofilm thickness was subjected to Periodic Acid Schiff (PAS) examination and epithelial thickness was subjected to HE examination, then observed under a microscope (400x magnification), then statistically analyzed (independent t-test for biofilm thickness and one-way ANOVA for epithelial thickness, $p<0.05$). As the result, biofilm thickness of ICAG was thicker than NTG. The epithelial thickness of HG was thicker than in other groups. No biofilm was found in group HG. Meanwhile, Shapiro-Wilk normality test and Levene's homogeneity test ($P>0.05$) was performed to biofilm and epithelial thickness. Significant differences existed between ICAG and NTG (biofilm thickness, $p<0.05$). There were significant differences between HG and ICAG and between ICAG and NTG ($p<0.05$). There was no significant differences between HG and NTG ($p > 0.05$). The thickest biofilm with the lowest epithelial thickness in tongue induced by *Candida albicans* ATCC-10231 in immunosuppression condition was found in ICAG.

Key words : Biofilm thickness, *Candida albicans*, epithelial thickness, corticosteroids, immunosuppressed.

How to cite : Dwi Andriani, Syamsulina Revianti, Agni Febrina Pargaputri, Deaniddo Kharisna, Ghina Rahmania Fikri and Rima Parwati Sari (2021) Biofilm and epithelial thickness in tongue of wistar rats (*Rattus novergicus*) induced by *Candida albicans* with immunosuppression condition. *Biochem. Cell. Arch.* **21**, 3267-3272. DocID: https://connectjournals.com/03896.2021.21.3267

INTRODUCTION

Candida albicans (*C. albicans*) are commensal microorganism that can turn into pathogens and the main causative agents of fungal infection is called oral candidiasis despite other types of *Candida* spp, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and other candida isolated from the surface of the oral mucosa (Bojang *et al*, 2021; William and Lewis, 2011; Mensana *et al*, 2018; Nugraha *et al*, 2018a). The change of *C. albicans* into a pathogen is caused by the host being under immunocompromised conditions such as cancer, chemotherapy, HIV infection, transplantation and long-term use of immunosuppressive drugs (Pathakumari *et al*, 2020; Wicaksono *et al*, 2020). In HIV/AIDS patient, oral manifestation related to candidiasis can be found such as liniear gingival erythema, angular cheilitis, chronic hyperplastic candidiasis, acute erythematous candidiasis, and pseudomembranous candidiasis due to declined Cluster of Differentiation 4 (CD4+) counts (Nugraha *et al*, 2017; Nugraha *et al*, 2019). Cheng *et al* (2017)

reported that 3.92% of inhaled corticosteroid users suffered from oral candidiasis. Van Boven *et al* (2015) noted an increase in the treatment of oral candidiasis in the inhaled corticosteroid users in the first year of use. Furthermore, Abdul Majid and Taher (2015), also reported a case of acute pseudomembranous candidiasis caused by the long-term systemic corticosteroid use (dexamethasone 10 mg/day).

The defence of mucosal immune system under normal conditions can inhibit *C. albicans* through a gradual process, such as pathogen recognition, cytokine production and immune cell phagocytosis (Zhou *et al*, 2021). However, this will be different in patients with immunosuppressed conditions. The use of corticosteroids in the long term can cause a suppression in immune system, such as a suppression in the activation of dendritic cells; a reduction in cytokines release by macrophages, B lymphocytes and antibody production; a number expansion of circulating neutrophils but with the inhibition of their apoptosis; an alteration of T-lymphocyte cytokines production and a cause for the host to be in an immunosuppressed condition (Abdul Majid and Taher, 2015). *C. albicans* colonies will proliferate and increase their virulence factors, then cause severe infection to death, especially in immunocompromised patients (Zhou *et al*, 2021).

The colonization of *C. albicans* on oral mucosa begins with *C. albicans* attachment to the epithelial side, and then they change their morphology into the invasive filaments (Naglik *et al*, 2011; Villa *et al*, 2020). The various morphologies of *C. albicans* in either oval or filamentous yeast forms also influence the interactions with the host (Moyes *et al*, 2010). Most *C. albicans* infections are associated with biofilm formation on the multiple surfaces. The transition of *C. albicans* from yeast to filamentous hyphae is central to its ability to form pathogenic biofilms (Villa *et al*, 2020). *Candida albicans* form the biofilm phase started with forming the microcolony, then producing EPS, starting to form a bilayer usually composed of yeast, germ tubes and/or young hyphae, and lastly the mature phase begins with thickening the EPS layer where the yeast and hyphae are present (Cavalheiro and Teixeira, 2018).

Symptomatic *C. albicans* infection is characterized by hyphae invasion of the oral epithelium leading to the release of inflammatory mediators by the host and clinically lthe ocal inflammation appears (Feller *et al*, 2014). In the immunosuppressed circumstances, there is a decrease in the rate of epithelial cell turnover. The integrity of epithelial tissue weakens and facilitates the penetration of candida into the oral epithelium. Epithelial alteration in the rats' tongues both in immunosuppressed conditions with or without *C. albicans* present. We also reported an increase in TLR2 expression in the oral candidiasis immunosuppression model compared with healthy rats (Andriani & Pargaputri, 2018). It is well known that innate immunity is generally mediated by pattern recognition receptors (PRR) such as Toll-like receptors (TLR), namely TLR2, TLR4, dextin1 and dextin-2, which are involved in recognition of *C. albicans* by monocytes and macrophages (Netea *et al*, 2015)

Meanwhile, the topical use of nystatin is the most common route of administration in dentistry because it has minimal systemic exposure. Nystatin also plays an important role in the prophylaxis of oral and systemic candidiasis in term and preterm newborns, infants, and immunocompromised patients (e.g., AIDS patients, cancer patients and organ transplant recipients), as it is reported to have a low incidence of drug interactions and its cost is acceptable, especially in the developing countries (Lyu *et al*, 2016). In Indonesia, the use of nystatin as an anti-fungal is an effective and affordable choice for oral candidiasis and also has great sensitivity to *C. albicans* (Anwar *et al*, 2012; Murtiastutik *et al*, 2019). Furthermore, Murtiastutik *et al* (2019) showed that nystatin is prescribed as an anti-fungal agent for the treatment of oral candidiasis in HIV patients. However, the doctors and pharmacist should be considered the long-term use of antifungal drug due to it can lead to drug adverse effect and drug resistency (Nugraha *et al*, 2018b). Considering the immunosuppression conditions have the potential to cause fungal infection led by *C. albicans* and the mechanism of damage by this fungus in this condition, this study intended to investigate biofilm and epithelial thickness in rats' tongues induced by *C. albicans* ATCC-10231 with immunosuppression conditions.

MATERIALS AND METHODS

This study was a true experimental with a post-test only control group design. It applied 16 Rattus Novergicus Wistar strain rats, aged around 12-weeks-old and weighed around 200-250 g, divided randomly into three groups with five replication each. These group were HG as the normal/ healthy rats group, ICAG as the oral candidiasis immunosuppressed rats group, and NTG as the oral candidiasis immunosuppressed rats group treated with nystatin. The applied sampling technique in this study was a simple random sampling technique. The eligibility of the study was approved by the Research Ethics Committee of the Faculty of Dentistry at Hang Tuah University, Surabaya (EC/016/KEPK-FKGUHT/VIII/2021).

The immunosuppressed condition was obtained by provisioning dexamethasone 0.5 mg/day and tetracycline 1%/day orally for seven days. On Day 8, the dose was reduced up to 10% for dexamethasone, and tetracycline was remained for 1%. On Day 8 to Day 20, the induction of *C. albicans* ATCC-10231on rats was applied with as much as 0.5 cc Mc. Farland 1 ($3x10^8$ CFU/mL) on the dorsum of tongues three times a week for two weeks. Nystatin in this study was administered to the surface of the tongue at the same time twice a day for two weeks. Then, the rats were then sacrificed and the tongue was then isolated (Andriani and Pargaputri, 2018; Andriani and Pargaputri, 2019; Andriani *et al*, 2019).

The measurement of biofilm thickness was completed with Periodic acid–Schiff (*PAS*) staining. It was carried out using a light microscope (Olympus) supported with an optilab viewer at 400x magnification. Biofilm thickness examination was done by measuring the thickness of the candida colony at the upper site of epithelium until the end of candida invasion (yeast or and hyphae forms).

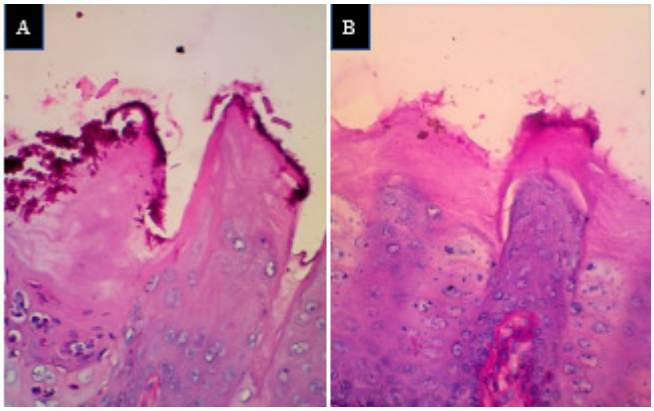

Fig. 1 : Biofilm in the epithelium of rats' tongue. A: ICAG: oral candidiasis immunosuppressed rats group; B: NTG: oral candidiasis immunosuppressed rats group treated with nystatin (400x magnification).

The measurements were performed in five selected fields of view using raster image software, then the average value was calculated (Meilawaty *et al*, 2020).

The measurement of epithelial thickness was concluded with Haematoxylin-eosin (HE) staining. Similarly with biofilm thickness, it was also carried out using a light microscope (Olympus) supported with optilab viewer at 400x magnification. Epithelial thickness examination was completed by measuring the thickness of epithelium tongue in rats from stratum corneum to stratum basalis. The measurements were done in five selected fields of view using raster image software, then the average value was estimated (Meilawaty *et al*, 2020).

RESULTS

The results expressed that biofilm thickness in rats' tongues induced with *C. albicans* ATCC-10231 under immunosuppression condition is shown in Figure 1. Figure 2 indicates that the biofilm thickness of ICAG group is thicker (62.46±4.21) than NTG group (23.36±1.12). In the healthy group, images were not displayed and measurement was not performed because there was no biofilm in the epithelium.

Furthermore, Shapiro-Wilk normality test had been conducted ($P>0.05$) showing normally distributed data and followed by Levene's homogeneity test ($P>0.05$). The independent t-test was held afterwards to see the differences between groups. There were significant differences between the ICAG group and the NTG group ($p < 0.05$).

Meanwhile, the epithelial thickness is shown in Fig. 3. Fig. 4 presents the epithelial thickness of group HG (135.83±8.94), ICAG (95.57±3.89) and NTG (134.56±2.08). HG group was thicker than other groups.

As a follow up, Shapiro-Wilk normality test had been

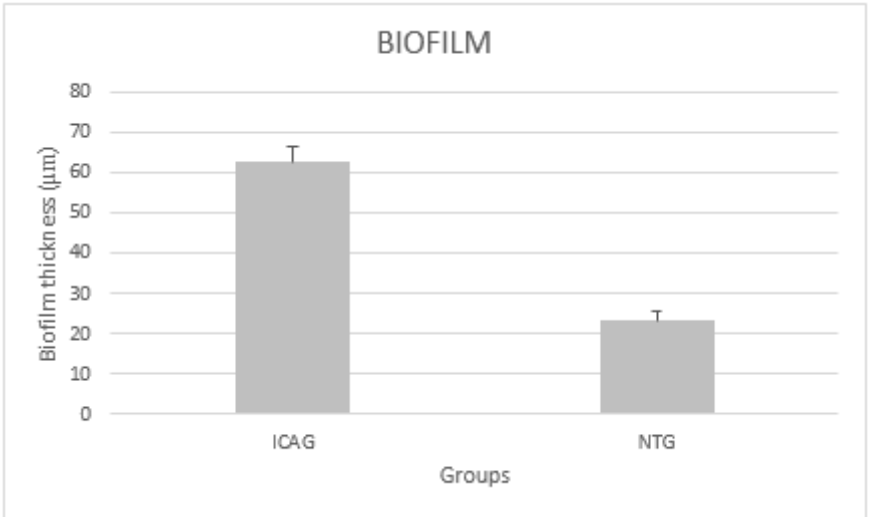

Fig. 2 : Means + standard deviations in ICAG group and NTG group (µm).

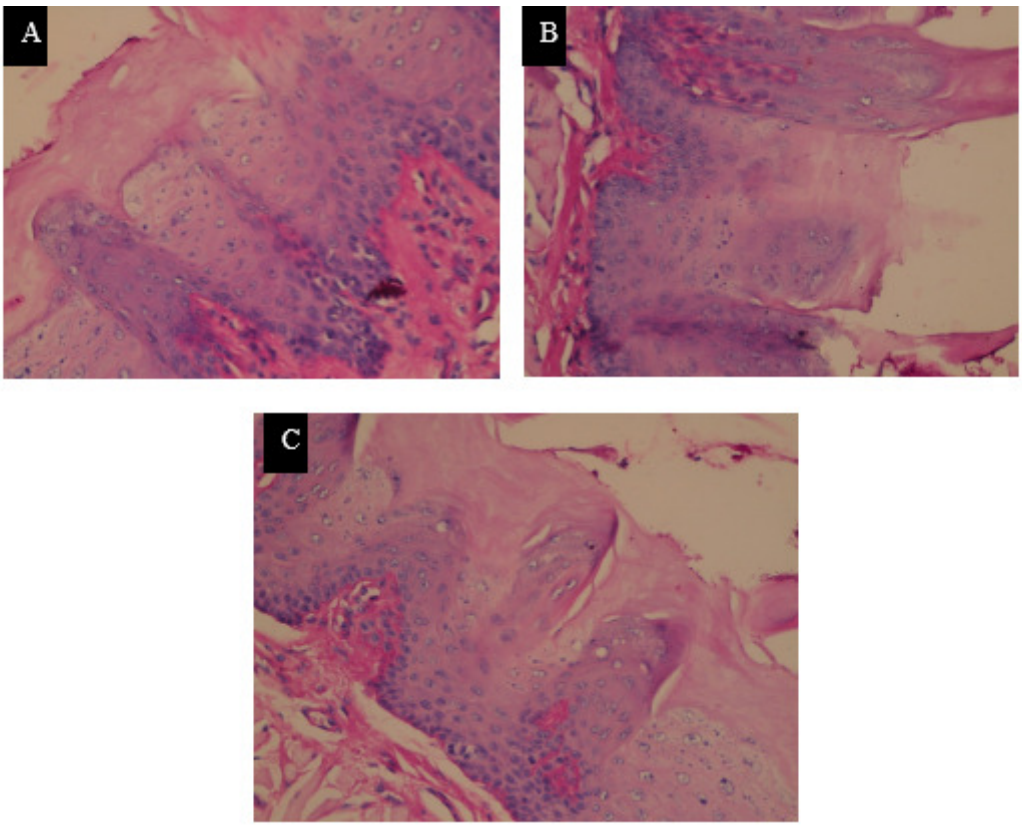

Fig. 3 : Epithelial thickness in the epithelium of rats' tongues. A: HG: Healthy group; B:ICAG: oral candidiasis immunosuppressed rats group; C: NTG: oral candidiasis immunosuppressed rats group treated with nystatin (400x magnification).

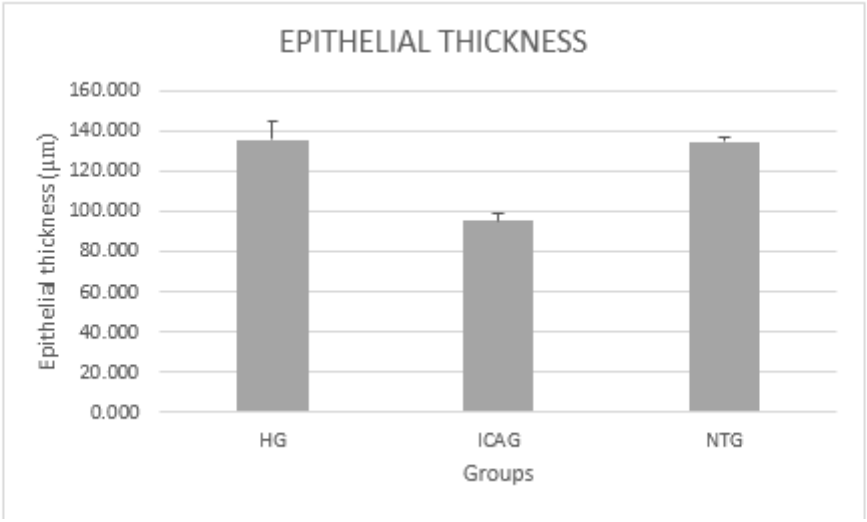

Fig. 4 : Means + standard deviations in HG group, ICAG group and NTG group (µm).

performed (P>0.05) showing normally distributed data. Then, it was continued by Levene's homogeneity test (P>0.05) and one-way ANOVA with post hoc Tuckey HSD was organized afterwards to see the differences between groups. Significant differences were presented between HG and ICAG group and ICAG and NTG group ($p < 0.05$). No significant differences were shown between the HG and NTG groups ($p > 0.05$).

DISCUSSION

In this study, the permanent biofilm layer could be found on the damaged tongue tissue in Wistar rats with oral candidiasis. It was because the tongue is the most sensitive organ in the oral cavity towards body changes. The biofilm of *C. albicans* was considered more susceptible to epithelial tissue damage in immunosuppressed conditions. This study revealed that the biofilm thickness increased in oral candidiasis within

the group with immunosuppression conditions. This proved that *C.albicans* colonize and invade to destroy the epithelium forming the ability of high resistance to any anti-fungal drugs. Correspondingly previous studies reported that after four days post sublingual infection with *C. albicans*, rats developed the oral candidiasis characterized by white plaques covering the tongue and other oral surfaces, while the histopathology of tongue tissue from the rats with oral candidiasis demonstrated an extensive penetration of epithelial tissue by the invasive *C. albicans* hyphae (Solis and Filler, 2012; Rahayu *et al*, 2018).

Biofilm development occurs in several phases and can be divided into different stages. In the initial stage, yeast cells attach and form the biofilm followed by cell aggregation and colonization. The formation of hyphae and pseudohyphae characterizes the intermediate stage. The final stage begins when the dispersing cells can be released from the biofilm to restart the process or serve as a reservoir of the infection (Mathe and Van Dijck, 2013; Cavalheiro and Teixeira, 2018; Costa *et al*, 2020). The increased levels of β-1,3 glucan are the feature of biofilm cells in fungal cell walls and act as the secreted forms (Tsui *et al*, 2016). The β-1,3 glucan, such as a matrix polysaccharide, critical contributes in the biofilm resistance to anti-fungals by inhibiting the drug diffusion process (Taff *et al*, 2012).

Compared to the treatment group with nystatin, biofilm thickness decreased in this group. This exhibited that the use of anti-fungal therapy had succeeded in reducing biofilms in this study. This is consistent with the previous study that there was a decrease in TLR2 expression in nystatin-treated oral candidiasis within the immunosuppression models (Andriani and Pargaputri, 2018). In general, nystatin works by disrupting the cytoplasmic membrane of fungal cells and interacting with ergosterol which is a component of the ergosterol cell wall which contributes in maintaining the integrity and function of fungal membrane enzymes. Furthermore, nystatin also causes damage to the proton gradient of cell membrane due to its ability to create pores leading to fungal cell death (Murtiastutik *et al*, 2019; Mohamadi *et al*, 2014). This mechanism generated successful therapy in this study.

Meanwhile, the paramenters in epithelial thickness as an indicator of this study was examines because it is one of the organs in the oral cavity lined with stratified squamous epithelium, an easy place for *C. albicans* colonization to develop and more susceptible to affect the epithelial tissue damage. In the oral candidiasis with immunosuppression conditions group, a decrease in the rate of epithelial thickness was indicated compared to the healthy group and treatment groups. This proved that there was an epithelial change in immunosuppression conditions, the integrity of the epithelial tissue weakens and it could facilitate the penetration of candida to the oral epithelium. Consistently, Andriani *et al* (2019) expressed that there was an epithelial change in oral candidiasis in immunosuppression models. Epithelial dysplasia occurred in the tongue of immunosuppressed rats and the *C. albicans*-induced group (Andriani and Pargaputri, 2019). Monteiro *et al* (2016) also stated that the wound healing in rat's skin under immunosuppressed conditions with dexamethasone induction was slower than the normal control group. Meanwhile, corticosteroid can be used to induce atrophy, thinning and fragility of the skin (Liu *et al*, 2013).

Moreover, the epithelial thickness of the treatment group with nystatin compared to the oral candidiasis under immunosuppression conditions were found thicker and not significantly different compared to the healthy group. This proved that nystatin succeeded in reducing *Candida albicans* colonies, thus, there were not any significant epithelial changes compared to normal group. Inconsistently, the previous study presented that there was moderate dysplasia in the tongue of nystatin-treated rats compared to normal group (Andriani *et al*, 2019). Dysplasia is interpreted as abnormal growth and it has a grading system for oral epithelial dysplasia from WHO which is not measured from the epithelial thickness (Ranganathan and Kavitha, 2019). Therefore, the results of this study are different from the previous study. The use of topical nystatin for two weeks has proven to be successful in preventing the growth of candida, therefore it can be seen that the thickness of epithelium did not decrease significantly. Additionally, the topical drugs absorbed into the oral epithelium are necessary for killing yeast hyphae growing within the tissue (Lyu *et al*, 2016). The investigation from Lyu *et al* (2016) exhibited that the use of nystatin administration for four weeks gives better clinical efficacy than two weeks of nystatin usage. However, observing the presence of colonies on the epithelium after treatment indicates that it need more evaluation for longer administration time or it is necessary to evaluate the use of other anti-fungal drugs.

CONCLUSION

This investigation indicated that the thickest biofilm in rats' tongues induced with *Candida albicans ATCC-10231* in immunosuppression condition was oral candidiasis in immunosuppressed rats group, while the thickest epithelial thickness was in the healthy group. Oral candidiasis in immunosuppression conditions had the

lowest epithelial thickness. Other examinations with specific markers and longer administration time are needed to confirm this oral candidiasis model.

ACKNOWLEDGMENTS

We want to thank Universitas Hang Tuah Surabaya and the students of Faculty of Dentistry, Universitas Hang Tuah Surabaya, who participated in the fieldwork.

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