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Microbial and histological analysis of rat model of oral candidiasis

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Abstract

Objective: This study aims to evaluate the infection after the treatment we provide, both microbiologically and histologically.

Material and Methods: Our male and female rats lowered their immune systems with the corticosteroid methylprednisolone. The rats’ drinking water was mixed with antibiotics to disrupt the balance of the oral cavity’s normal flora. To facilitate C.albicans exposure and avoid loss of C.albicans, rats were previously injected with chlorpromazine HCL.

Results: The microbiological evaluation showed that in male and female rat models, the number of fungal colonies on oral mucosal swab cultures was significantly higher at five and eight days after exposure to putrefactive control mice. Histological evaluation showed that in model rats, the number of hyphae that penetrated the dorsum of the tongue was higher than in control rats five days after exposure.

Conclusion: We used is proven to produce oral candidiasis model rats, both male, and female.

Keywords: Histological evaluation, Microbiological evaluation, Oral candidiasis, Rat model
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Introduction

In the last few decades, there has been a rapid increase in the prevalence of fungal infections, including Candida albicans, the most common opportunistic yeast found in the human mucosa. This is in line with the rise of various predisposing factors for C. albicans infection, such as; diseases that lower the immune system, including HIV infection, massive use of antibiotics, use of chemotherapy/radiation, and elevation of life expectancy.1 Fungal infection is a troublesome problem because of the limited type of antifungal drugs and their side effects.2 In the last few years, the resistance of C. albicans to the existing antifungal was found.3 In patients with a weakened immune system, the infection can be chronic and require long-term therapy. On the other hand, an antifungal drug that is effective for severe conditions and is safe for long-term use has not been found.

C. albicans, discovered about 150 years ago, is humans’ predominant opportunistic commensal fungus. As a commensal, C. albicans is present in the digestive tract, mouth, skin, and female reproductive tract of at least 70% of healthy adults and has been in the human body since infancy. C. albicans is one of about 200 species in the genus candida but is responsible for up to 75% of all candida infections.4 Candidiasis describes a group of fungal infections of candida species, especially C. albicans. Superficial candida infections often occur in the oral cavity, reproductive organs, gastrointestinal tract, and skin. In addition to superficial infection, C. albicans can cause life-threatening systemic infections. These infections can occur in the heart, eyes, intra-abdominal, joints, bones, and brain membranes.5 Mucosal infections caused by C. albicans are the most common fungal infections.6

Various studies have been conducted to discover alternative antifungal drugs, including natural ones.7 The research uses animal models that can represent infectious conditions in humans. Accurate experimental animal models are necessary because they determine the success of a study to be applied and avoid wasting resources. The experimental animal model that is widely used in research is the mouse model.8 The existing protocol to make the candidiasis animal model use mice. Mice have limitations due to their relatively small size (about 25 g), short lifespan, and only about 2 ml of blood that can be drawn.9 In this study, we will extrapolate existing research, apply it to the Wistar rats, and evaluate the microbiologic and histopathologic of the infection. Wistar rats were chosen because they are larger in size and body weight (300 grams) and have a relatively long life (2.5 to 3.5 years). These factors make it possible to observe effectiveness and side effects better.10 This mouse model is also preferred in exploratory research on drugs derived from extracts of natural ingredients because they have a larger stomach.

This study aimed to examine the infection that occurred through microbiological and histological evaluations in female and male oral candidiasis rat

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models. The microbial assessment was carried out by counting the number of fungal colonies obtained from swab cultures on the entire oral mucosa. One sign of infection with opportunistic microorganisms is an increase in these microorganisms. The histological evaluation of C. albicans infection was carried out by observing and counting the hyphae that penetrated the mucosa. The number of hyphae present in the epithelium is used as a marker of the severity of the infection.

**Material and Methods**

C. albicans was obtained from and identified at the Laboratory of Oral Biology FKG, Universitas Hang Tuah. Rats are placed in cages with an ambient temperature of 26 - 29°. One cell was used to keep four rats. The rats used in this study have been declared healthy at Klinik Hewan Dinas Peternakan Kab. Jember. All research procedures had been approved by the Komite Etik Penelitian Kesehatan (KEPK) FKG Universitas Jember with approval letter 11280/UN25.7/KEPK/DL/2021.

The research was an experimental laboratory with a post-test-only control group design, using female and male rats. Male (CM0) and female (CF0) control groups were healthy rats without treatment. The treatment groups were male rats (TM1 and TM2) and female rats (TB1 and TB2). Each group consisted of 6 rats.

Since the first day, the drinking water of all of the treatment groups (TM1, TM2, TF1, and TF2) was added with Tetracycline HCl 500 mg/L to reduce the population of the bacteria in the oral cavity, which can inhibit the growth of C. albicans. On the second and fourth days, the rats in the treatment group were injected with methylprednisolone 6 mg subcutaneously to suppress the immune response of the rat. On the 3rd day, using a micro brush, 0.3 ml C. albicans suspension (9.4 x 10^7 cells/ml) was inoculated into the oral cavity of the rat. Before this procedure, the rat was injected with Chlorpromazine Chloride 0.75 mg/kg BW intramuscularly. The microbial and histological evaluation was done on the 5th day (T1 and T1B groups) and the 8th day (the T2 and T2B groups). This procedure was adapted and modified from the method used by Takakura et al.11

The microbial evaluation started with swabbing all oral cavity surfaces using a sterile cotton swab. Then the tip of the cotton swab was cut and put into a physiological saline solution tube. The tube was then incubated at 37°C for 24 hours. After five times dilutions, the 0.1 mL suspension of C. albicans was cultured in Sabouraud’s Dextrose Agar media containing chloramphenicol (SDA-C) and then stored in an incubator at 37°C for 24 hours. The number of candida colonies in the culture was counted using the colony counter. Culture characteristics on the SDA, C. albicans, show white colored, smooth, and yeast-like appearance.12

The histological evaluation of infection was done by observed of the hyphae in the mucous membrane of the dorsum tongue. The rat tongue was cut and immersed in 10% formalin buffer solution to prevent autolysis, maintain morphology, and prevent fungal and bacterial growth, then dipped in paraffin to be paraffin block. Paraffin blocks were cut 5 mm in thickness and then dehydrated with 70% alcohol for 15 minutes, then 80% for 1 hour, 95% for 2 hours, and 100% for 3 hours. After that, the clearing process was carried out using xylol three times for 1 hour, 2 hours, and 2 hours, respectively. Tissue staining using Periodic Acid Schiff (PAS) to verify the fungal filament level and depth of tissue penetration.13 Histological preparations were observed using a binocular light microscope with a magnification of 400x.

**Results**

C. albicans is a commensal opportunistic microorganism in the human oral cavity. Changes in the oral immune system can drive the change from commensalism to pathogens. The virulence factors of C. albicans include cell surface adhesins, proteolytic enzymes, morphological changes, and the development of drug resistance. In the oral cavity, the balance between C. albicans and bacteria and local innate immune defences play a central role in maintaining Candida’s commensal state. In particular, in addition to preventing Candida attachment to epithelial cells, saliva is enriched with anti-Candida peptides, which are considered part of the host’s innate immunity. T helper type 17 (Th17) adaptive immune response is mainly involved in mucosal host defense, controlling the initial growth of Candida and inhibiting subsequent tissue invasion.14

The cultures of candida in SDA-C from the oral swab in the treatment groups looked denser than the control group in both males and females figure 1.

The mean ± standard deviation CFU of the male control group was 0.168 ± 0.1235; in the treatment group 1 (TM1) was 5.587 x 10^5 ± 3.3353 and in the TM2 group was 3.628 x 10^5 ± 1.8222. In the female rat mean ± standard deviation of the control group was 0.453 x 10^5 ± 0.2426; the treatment group 1 was 9.778 x10^5 ± 4.258626, and the treatment group 2 was 3.057 x 10^5 ± 0.963487. The histogram of these mean CFU is illustrated in figure 2.

The normality and homogeneity analysis of the quantity of CFU showed that the data was normal...
and homogeneous (p ≥ 0.05). The differences between groups were analyzed by one-way ANOVA followed by the LSD test. The ANOVA test results showed a difference between groups (p ≤ 0.05). The differences between groups were analyzed by one-way ANOVA followed by the LSD test. The ANOVA test results showed a difference between groups (p ≤ 0.05). The results of the LSD test, there were significant differences between the control group, treatment group 1, and treatment 2 in both male and female rats. This means that on the 5th and 8th days after inoculation of candida, the number of colonies was higher than the control group. On day 8, the average number of colonies was lower than on day 5. Between the males and females groups, the number of CFU in the female group was higher than in the male group in treatment group 1. (p ≤ 0.05).

Observation of histological preparations to identify hyphae that invaded the mucosa using a binocular light microscope with 400x magnification. The histologic feature of hyphae on the mucosa of the dorsum of the tongue of the rat groups can be seen in figure 3.

The hyphae in the mucous membrane were counted per microscopic field, and scores ranged from 0 to 4. The score was 0 if there were no hyphae, score 1 if 1 to 5 hyphae; score 2 if 6 to 15 hyphae; score 3 if there were 16 to 50 hyphae, and score four if more than 50 hyphae. The range of the score in each group showed in figure 4.

Further analysis of the data score was performed with Kruskal-Wallis, followed by Mann Whitney. The results of the Kruskal-Wallis test showed a difference in the rat groups. The results of the Mann-Whitney test showed that in both male and female rats, there was a significant difference between the control and treatment groups 1. The number of hyphae in treatment group 1 was significantly higher than in the control group, but the mean of treatment group 2 was not substantially higher than in the control group. There was no significant difference between male and female rats in the control group, treatment group 1, and treatment group 2 (p ≤ 0.01).

Discussion

Animal models that are valid and scientifically proven to represent conditions of opportunistic infections greatly determine the study’s success in exploring materials for therapy. The method for obtaining an experimental animal model of opportunistic infection is more complex because the immune system in the experimental animal can cope with the exposure given. Our study showed a very large (almost 4-fold) increase in Candida colonies on day five after C. albicans inoculation. The injection of chlorpromazine before inoculation caused the rat to be sedated and prevented vomiting. This condition makes the procedure of candida inoculation easier to be done.
On the 8th day, the number of colonies was lower than on the 5th day but was still significantly higher than the control. The immune response of the rats, which was reduced by the injection of corticosteroids (methylprednisolone/MP), combined with adding an antibiotic tetracycline in drinking water which can eliminate bacteria in the oral cavity, supports the growth of C. albicans. MP injection was injected subcutaneously because it can slowly absorb over a period of time. That causes suppression of the immune response will occur longer. Methylprednisolone inhibits cell-mediated immunologic functions, especially those lymphocyte-dependent.

On the 8th day after exposure, the number of swab colonies was lower than on the 5th day. This was probably because the MP levels in the rat’s blood began to decrease. This is in line with previous research on rat models using the same procedure; IL-17 values increased on day 8.

The difference in the number of fungal colonies in the swab results between male and female rats occurred in treatment group 1. The mean number of colonies in female rats was significantly higher than in male rats. The probable reason is that the effect of steroids on female rats is higher than that on male rats. Because estrogen and progesterone are also steroid hormones, they can affect or increase the work of methylprednisolone.

The core of C. albicans pathogenesis is its ability to undergo morphologic switching. In infection conditions, C. albicans changes to the most pathogenic form, the hyphae. Hyphae is associated with expressing hypha-associated virulence factors that aid in adhesion to and invasion into host cells. A critical property of hyphal cells is their ability to directional growth in response to contact with a surface (thigmotropism), allowing the fungus to invade intercellular junctions specifically. In addition to active penetration, which is a fungal-driven process, another complementary mechanism utilized by C. albicans for host cell invasion is endocytosis, a passive fungal-induced but host cell-driven process whereby lytic enzymes and invasins expressed on hyphae bind to and degrade E-cadherin and other inter-epithelial cell junctional proteins, enabling the organism to penetrate between epithelial cells.

The transformation of C. albicans from yeast to hypha can help fungi escape the phagocytosis of macrophages, increasing the likelihood of invading host tissues and causing more significant damage. Hyphae cells have tubular, multicellular forms, which can be induced by a temperature of 37°C, N-acetyl glucosamine, embedding matrix, hypoxia and hypercapnia, alkaline pH in vitro, involvement in biofilm formation, and the ability to grow thigmotropically. The width of hyphal cells is about 2.0 µm on most media.

In this study, the score for the number of hyphae found to penetrate the epithelium of the dorsum of the rat tongue five days after treatment was significantly higher than the control group, but no significant difference between the control and the group was observed eight days after treatment. This is probably because on the 8th day, there was an increase in the immune response, which could eliminate the hyphae in the rat tongue mucosa. As explained above, in rat models made using the same method, there was an increase in IL-17 on day 8. IL-17 is a crucial cytokine in the body’s defense against C. albicans. If the study uses this rat model, more extended observations are needed, so the authors suggest repeating subcutaneous MP injections on day 8. The hyphal score data in all groups did not show significant differences between male and female rats. The severity of the infection seen from the hyphae that penetrated the oral mucous membrane in male and female mice did not differ significantly. There are no differences in the immune response to fungi in both sexes. Estrogen and Progesterone in women affect the number of hyphae penetrating the oral mucosa.

**Conclusion**

Inoculating C. albicans into male or female rats injected with steroids and given drinking water mixed with antibiotics can produce an experimental animal model of oral candidiasis. This experimental animal model can be used in studies to explore the therapy of oral candidiasis.

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Conflict of Interest
The authors report no conflict of interest.

References

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