Evaluation of rat model oral candidiasis mucosal response immune

Erna Sulistyani, Iin Eliana Triwahyuni*, Happy Harmono**, Almaratus Sholehah***, Syefira Putri Prameswari***, Zahrah Mayang Sari***, Brigitta Damai Perwitasuci***, Widya Nurfitriastuti Wulandari****

**Oral Medicine Dept. Faculty of Dentistry, Jember University*
*** Biomedical Dept. Faculty of Dentistry, Jember University*
**** Faculty of Dentistry, Jember University*

Received 28 January 2023; 1st revision 9 May 2023; 2nd revision 18 December 2023; Accepted 21 December 2023; Published online 30 December 2023

Keywords:

Wistar rat; oral candidiasis; immune system; animal models

ABSTRACT

Background: There has been a high elevation of oral candidiasis's prevalence of oral candidiasis recently, but the choice of antifungal drugs is minimal and has yet to make significant progress. The animal model of oral candidiasis is a crucial factor in exploring antifungals. In our previous study's oral candidiasis rat model, we found an increase in the sedimentation rate and a decrease in infection microbiologically and histologically on day 8 compared to day five after inoculation. On the other hand, blood plasma levels of IL-17, as the main cytokine in fungal infections, are kept at a low level. The study aims to prove the increase in the immune response in the oral mucosa of an oral candidiasis rat model on the eighth day after inoculation of C. albicans.
Methods: The sample consisted of 2 groups: healthy Wistar rats as a control and Wistar Rat rats treated as an oral candidiasis rat model, i.e., immune systems were lowered by injection of steroids, given drinking water containing antibiotics, and inoculated with C. albicans. Eight days after inoculating C. albicans, the tongue tissue was cut, placed in formalin buffer, and processed into paraffin blocks. The immune response was evaluated by counting the number of visual field inflammatory cells on the dorsum of the rat tongue.
Results: on day 8, the number of inflammatory cells in the dorsal tongue mucosa of oral candidiasis rats was higher than that of healthy rats. **Conclusion**: The immune cells in the oral sub-mucous membrane of the rat model of oral candidiasis increased eight days after inoculation, a sign of an increased immune response in the experimental animal model.

doi: http://dx.doi.org/10.30659/odj.10.2.172-179
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Odonto : Dental Journal accredited as Sinta 2 Journal (https://sinta.kemdikbud.go.id/journals/profile/3200)
How to Cite: *Sulistyani et al*. Evaluation of rat model oral candidiasis mucosal response immune. Odonto: Dental Journal, v.10, n.2, p. 172-179, December 2023

INTRODUCTION

In the last decades, there has been a high elevation in the prevalence of infections by fungus, especially the opportunistic fungus C. albicans. The increase is simultaneously caused by the rise in various predisposing factors for C. albicans infection, such as diseases that reduce the immune system, especially HIV infection, use of antibiotics, chemotherapy/radiation, and prolonged life expectancy.[1] This is complicated because the types of antifungal drugs are limited, have many significant side effects, and increase resistance to antifungals.[2] In patients with a low immune system, the infection can become chronic, requiring long-term therapy. Invasive fungal diseases (IFD) remain a serious and under-recognised cause of illness and death worldwide despite advances in medical technology and interventions in patients in intensive care units or people with cancer or other immunocompromised diseases.[3] The emergence of modern diagnostic and therapeutic techniques is the cause of the increasing frequency of nosocomial infections, especially by the fungus Candida spp. Candida spp. infection is a major cause of morbidity and mortality in hospitalised patients, especially those with very severe disease.[4] Candida albicans also has a close relationship with caries. Candida albicans can be one of the microorganisms that cause caries because it is able to tolerate and create acidic conditions. The utilisation of glucose produces pyruvic acid and acetic acid, which can dissolve dental hydroxyapatite, resulting in dental caries.[5] Until recently, antifungal drugs that are effective for severe infections and safe for long-term use have not been obtained.[6]

Antifungals consist of polyene groups such as Nystatin and Amphotericin B or azole groups such as Clotrimazole, Fluconazole, and Ketoconazole. Administration can be done topically, but if needed, in cases where hyphae have invaded the tissue under the skin or in severe cases, antifungals must be given systemically. These drugs almost all have side effects ranging from nausea, vomiting, and diarrhoea to kidney and liver diseases.[7] Oral candidiasis in patients with low body resistance requires large doses of antifungals for a long time, so that these side effects can increase morbidity and mortality; besides that, there is much evidence of resistance to existing antifungals.[28] Appropriate research for the exploration of oral antifungal drugs is urgently needed.

Many studies have been conducted to discover alternative antifungal drugs, including natural ones.[9] Various in vitro studies have been carried out to overcome C. albicans infections, including research with temulawak extract, which has been proven to reduce the number of C. albicans in acrylic resin.[10] Experimental animal models are an essential factor in new drug research. It is necessary to develop an accurate experimental animal model to provide a standard tool that can be controlled and manipulated to obtain data on the etiopathology, diagnosis, and management of oral candidiasis.[11] Animal models have been an essential tool since the beginning of scientific discovery. Experimental animals are currently indispensable in biomedical research that contributes to our understanding of gene function, aetiology, mechanisms of various diseases, and the effectiveness and toxicity of drugs and chemicals.[12] The selection of any model, especially animal models for research, should be based on the following considerations: 1) suitability as an analogue, 2) transferability of information, 3) genetic uniformity of the organism, if applicable, 4) background knowledge of the biology of the animal, 5) cost and availability, 6) generalizability of results, 7) ease and adaptability for experimental

manipulation, 8) ecological consequences, and 9) appropriate in ethical.[13]

C. albicans is an opportunistic microorganism, kept as a commensal organism by the body's defence system.[14] Therefore, animal models of oral candidiasis cannot be created only by exposing C. albicans to the oral cavity. One of the in vivo studies of oral candidiasis used rats whose immune system was lowered by being given tetracycline and dexamethasone orally for 14 days and given 0.1 ml of C albicans suspension on the fourth day.[15] The rat model of oral candidiasis we made in previous studies showed infection through microbiological and histopathological evaluations on the fifth and eighth day after C. albicans inoculation.[16] On day 8, there was a decrease in infection compared to day 5, but on analysis of the IL-17 plasma level, it remained lower than normal.[17] This is controversial because IL-17 is a key cytokine in the defence against fungal infections.[17] In this study, we directly evaluate the immune response in the infected area, the oral mucosa of the rat model. This evaluation needs to be done so that the infection can run according to the time required for a study. Considering that C. albicans is an opportunistic microorganism if the immune response is seen to increase and the technique is not modified, the research results will also be inaccurate. The Rattus norvegicus, particularly the Wistar Rat, is the most commonly used animal model in biomedical research. This animal can represent the biological system of mammals, making it a suitable animal model in pre-clinical studies.[18]

RESEARCH METHOD

This study used healthy female Wistar rats aged 1.5 months, weighing approximately 150 grams. All treatments in rats have received approval from the Health Research Ethics Committee (KEPK) FKG University of Jember with approval letter 1679/UN25.8/KEPK/DL/2022. The treatment to produce the rat model oral candidiasis was initiated by adding Tetracycline HCL 500 mg/L in drinking water until treatment's end. The addition of antibiotics to drinking water aims to reduce the number of oral florae that can inhibit the growth of C. albicans. On the same day, rats were injected with 80 mg/kgBW Methylprednisolone SC to lower the rats' immune systems, and this injection was repeated a day after inoculating C. albicans. The inoculation of C. albicans was performed after the rats were sedated with 0.05 mg/kgBW Chlorpromazine IM injection on both femurs. The suspension of *C. albicans* ATCC 10231 (3,1 x 10^6 cells/rat) was spread in the oral mucous membrane of the oral cavity with a small brush. Sedated rats are easier to handle, and inoculation can be performed accurately. Chlorpromazine can also prevent vomiting. In our observations, rats were sedated for up to 3 hours in that dose, which prevented C. albicans in the rat's oral cavity from subsiding due to eating, drinking, or other activities. In the following days, the rats were kept and fed as usual. The sacrifice procedure of rats according to the rules Animal Euthanasia Policy of the University in St Louis, USA. The rat was anesthetised with a Ketamine-Xylazine cocktail 0,2 mL/200 gmBW, followed by exsanguination. Ketamine Xylazine cocktail is prepared by mixing 10 mL of ketamine (100 mg/mL) with 1 mL of xylazine (100 mg/mL).[19,20]

After euthanasia, the rat's tongue was cut, put in a sealed bottle containing 10% formalin buffer, and then processed into a formalin-fixed, paraffin-embedded (FFPE) block of tissue. The next step was to make the slide by cutting paraffin-embedded tissue using a rotary microtome. Three tissue sections from the same paraffin block are placed on one slide. The number of the three types of cells was observed under a microscope with a

magnification of 400 times on slides stained with Hematoxyline-eosin. In addition, because of the critical role of macrophages in the body's defence against fungi, the number of macrophages was counted in the immunohistochemical staining slide using the antibody CD68(KP1) sc-20060 from Santacruz Biotechnology. The tongue of healthy female rats with the same characteristics was used as a control.

RESULTS

The stained slides were evaluated under a microscope with 100x magnification to identify suitable areas of the preparation for the high-power examination, i.e., well stained, not too thick or thin. Furthermore, calculations were carried out on three visual fields in suitable areas with a magnification of 400 times. Two people counted the cells, and the number of cells in the visual field was averaged. All inflammatory cells were counted in Haematoxylin-eosin-stained tissues without determining the cell type. We could not accurately determine the type of inflammatory cells in the tissue staining by haematoxylin-eosin (HE). The inflammatory cell was determined by the cells laid out against a background of mucosal tissue and are not organised as connective, adipose, or epithelial tissue. On the slide with IHC staining, the cells counted were brownish, indicating CD68 expression. The results of HE-staining on the tongue dorsum in oral candidiasis model rats on day eight and healthy control rats can be seen in Figure 1.

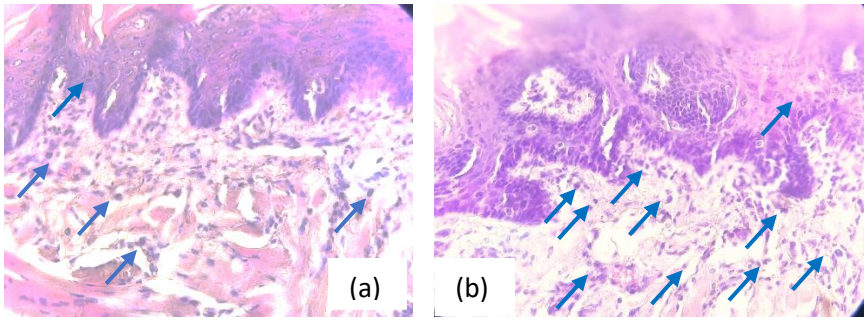

Figure 1. Histological picture of dorsum tongue (HE 400X) A. Healthy Rat, B. rat model of oral Candidiasis 8 days after inoculation of C. albicans. The arrows point the inflammatory cell

The mean histogram and standard deviation of the inflammatory cell count with a field of view of 400x are shown in Figure 2.

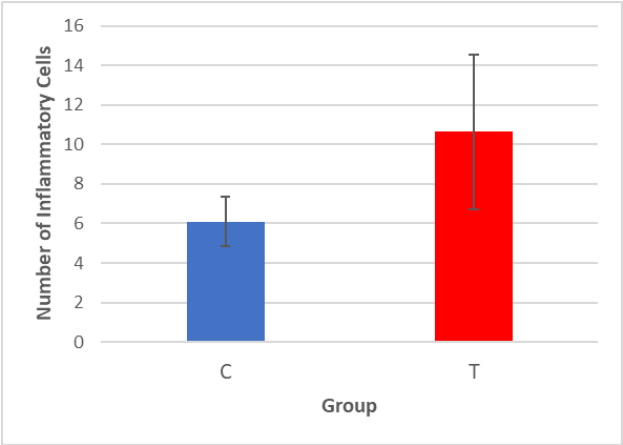

Figure 2. The histogram of the mean and standard deviation of inflammatory cell count in the dorsum tongue of the rat. The (C) is the control group, and the (T) is the rat model of oral candidiasis 8 days after inoculation of C. albicans

The results of immunohistochemical staining with CD68 antibody are shown in Figure 3.

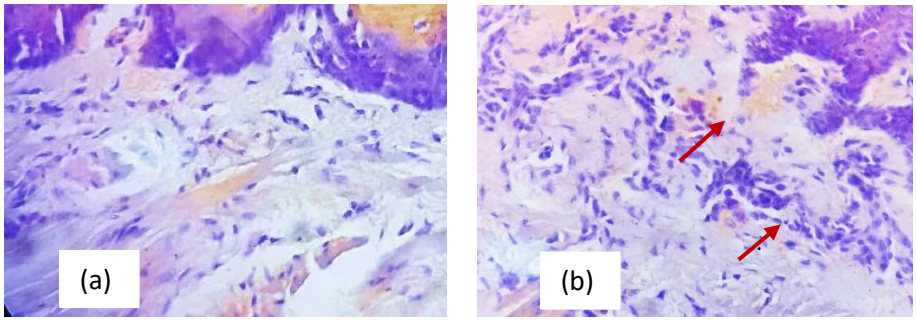

Figure 3 Expression of CD 68 in the slide of dorsum tongue in (a) healthy rat (b) rat model of candidiasis on the eighth day (IHC staining using antibody CD68-sc 20060, Santacruz Biotechnology, 400x)

The mean number of CD 68 expressions and their standard deviations are shown in Figure 4.

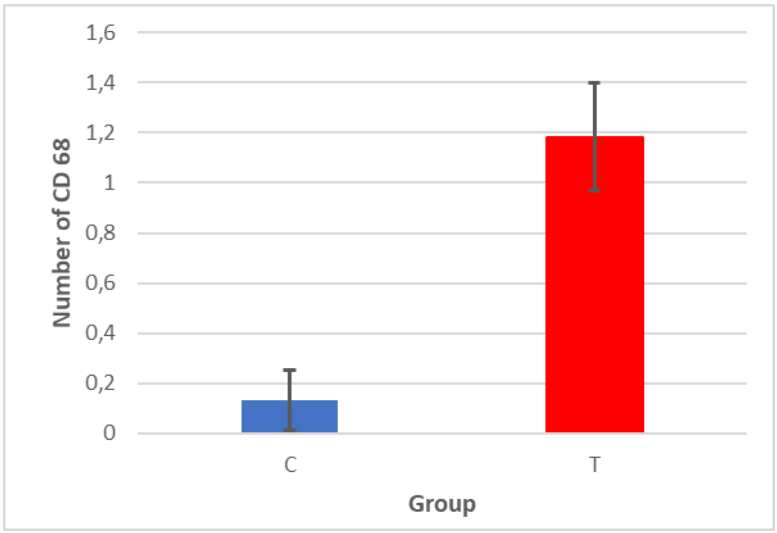

Figure 4. The histogram of the mean and standard deviation of expression CD68 count in the dorsum tongue of the rat. (C) control/healthy rat (T) rat model of oral candidiasis on the eighth day after inoculation of C. albicans

The number of inflammatory cells and expression of CD 68 were analysed using the SPSS 26 software. After the data was confirmed as normal and homogeny by the Shapiro-Wilk and Levene's tests, the difference test was carried out with the independent t-test, resulting in a significant difference between groups. From the results of this analysis, considering the histograms in Figures 2 and 4, it can be concluded that the number of inflammatory cells and expression of CD 68 on the dorsum of the rat tongue oral candidiasis was higher than in control rats.

DISCUSSION

The limitation of the range for antifungals has encouraged researchers in recent decades to look for alternative antifungal targets that have excellent effectiveness as an antifungal and minimal side effects. There were many reports of new potential antifungals, but applying research results for treating patients is a long process, and most of these agents fail.[21] One of the possible causes is inaccuracy in the manufacture of candidiasis animal models. As previously explained, making disease models due to opportunistic microorganisms requires more complex actions and evaluations.

Experimental animal models are used in research in the health sector because they have biological similarities and can be manipulated to represent the disease in humans. Animal models are similar to humans in anatomy, physiology, or response to pathogens. The results of these animal model studies can be extrapolated to understand human physiology and disease. Experiments can be carried out more practically and do not violate human ethics using animal models.[22]

This study showed that the number of inflammatory cells and expression of CD68, a marker of macrophages, in the oral candidiasis rat model on day eight after inoculation of C. albicans were higher than in healthy rats. These results indicate an increase in the immune response in the rat model of oral candidiasis. This result follows the results of previous studies that on the eighth day, there was a decrease in infection both microbiologically and histologically and an increase in the sedimentation rate of the oral candidiasis rat model. The elevated erythrocyte sedimentation rate may indicate increased inflammation-sensitive plasma proteins (ISPs).[17] The results of this study proved that even IL-17 levels in the oral candidiasis rat model remained at a low level on the eighth day, but the mucosal immune response in the rat model increased. Even though IL-17 is the main cytokine of the body's defence against fungi, it is well known that cytokines in the body will work together synergistically in fighting infections, including fungal infections.[23] A study that compared the levels of 14 types of cytokines in 41 fungemia patients and 57 control patients (negative blood culture fungi) showed that in patients with fungemia, the serum levels of IFN-γ, TNF-α, CCL4, IL-6, IL-8, IL-10, IL-12p70, and IL-17 were significantly higher compared with the control group.[24]

The increased inflammatory cells in the oral candidiasis rat model's oral mucous membrane are due to increased chemokines, particularly CCL3. CCL3 is important in the acute inflammatory response to *C. albicans* corneal infection.[25] CCL3 collaborate with CCL4 to attract macrophages, monocytes and neutrophils. Several studies have proven that monocyte dendritic cells, fibroblasts, and vascular smooth muscle cells secrete CCL3. INFα, TNFα, and IL-1β can stimulate secretion of CCL3. [26] From this fact, we can conclude that although IL-17 is the main cytokine, it does not mean that if IL-17 is blocked, the immune response in the oral candidiasis rat model cannot be increased.

In this study, the rat model of oral candidiasis was injected with Methylprednisolone 80 mg/kg BW subcutaneously 2 times, i.e. a day before and after C. albicans inoculation. The site injection at subcutaneous tissue is the intended order for the absorption into the blood to occur slowly. Because the subcutaneous tissue contains much fat, it does not have many blood vessels.[27] Methylprednisolone suppresses the immune response by suppressing the production of proinflammatory cytokines and increasing the synthesis of anti-inflammatory cytokines. After crossing the cell membrane by passively diffusing, Methylprednisolone binds to intracellular glucocorticoid receptors. This methylprednisolone receptor complex then enters the nucleus of specific DNA sequence cells, mainly by blocking the function of transcription factors, such as nuclear factor-kappa-B (NF-kB), and blocking proinflammatory gene promoter sites so that the production of proinflammatory cytokines will be inhibited, resulting in a decrease in the inflammatory response. In addition, this complex triggers the expression of anti-inflammatory genes, thereby increasing the production of anti-inflammatory cytokines.[28]

The increase in the immune response indicates that the suppression of the immune response has been significantly reduced. The elevation of immune response is probably due to the reduced levels of Methylprednisolone in the blood. Unfortunately, our study did not measure methylprednisolone blood levels. For further research other than this, it may be possible to measure levels of chemokines and other cytokines so that this phenomenon can be explained and the immune response to candida can be more broadly understood.

Our research also has limitations; the rats used as the animal model need a certificate proving their homogeneity, and the health history of the rats from infancy until they are used in the study is not known with certainty. Counting inflammatory cells in the field of view with a microscope has many weaknesses because the tissue observed only a small part and may not represent the whole condition. The results also depend on observers' interpretation, although we try to eliminate this using three observers.

The consequence of the results of our research is that if the protocol for making rat models of oral candidiasis is used in the exploration and evaluation of antifungal therapy that requires observation for a long time, then we suggest lowering the immune system with another injection of Methylprednisolone before the eighth day after inoculation**.**

CONCLUSION

There was an increase in inflammatory cells, including macrophages, eight days after inoculation of C. albicans in the mucous membrane dorsum tongue of the rat model of oral candidiasis by Sulistyani et al. 2022.

ACKNOWLEDGMENT

We are grateful to the Lembaga Penelitian dan Pengabdian Universitas Jember for financial support for our research. We also would like to thank the Veterinary Laboratory of the Biomedical Department, the Faculty of Dentistry, Jember University, for support in the treatment of experimental animals, and the Biomolecular-Biochemistry Laboratory, Faculty of Medicine, Brawijaya University, for assistance in assistance and support in the process of measuring variables.

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