



# Oral Candidiasis in Immunosuppressed Wistar Rats (*Rattus norvegicus*) Post Dexamethasone Injection at 7.2 mg/kg and 16 mg/kg Doses

Candidíase oral em ratos Wistar imunossuprimidos (*Rattus norvegicus*) pós-injeção de dexametasona com doses de 7,2 mg / kg e 16 mg / kg

Ucy Nur HAMIDA Al ABRORI<sup>1</sup>, Nurina Febriyanti AYUNINGTYAS<sup>2</sup>, Diah Savitri ERNAWATI<sup>2</sup>, Hening Tuti HENDARTI<sup>2</sup>, Meircurius Dwi Condro SURBOYO<sup>2</sup>, Desiana RADITHIA<sup>2</sup>

1 - Faculty of Dental Medicine – Universitas Airlangga – Surabaya – Indonesia.

2 - Department of Oral Medicine – Faculty of Dental Medicine – Universitas Airlangga – Surabaya – Indonesia.

## ABSTRACT

**Objective:** Oral candidiasis is an infection that occurs in the oral cavity and is caused by candida species, often *Candida albicans*. This infection commonly occurs in a condition of immunosuppression caused by dexamethasone. Due to the side effects of antifungal therapy, developing a standardized immunosuppressed animal model to induce oral candidiasis for new therapies is required. The aim of this study is to observe oral candidiasis in immunosuppressed Wistar rats post dexamethasone injection at 7.2 mg/kg and 16 mg/kg doses. **Material and Methods:** Twenty-one Wistar rats were divided into three groups: control group, treatment group 1 (injected with dexamethasone at a concentration of 7.2 mg/kg), and treatment group 2 (at a concentration of 16 mg/kg) for five days. Immunosuppression status was observed by leukocyte count and all the subjects' palates were inoculated with *C.albicans* 0.1 ml of  $15 \times 10^8$  UFC/ml 24 hours later. The subjects' tongues were observed and confirmed by laboratory examination on day 10. A statistical analysis was performed using one way ANOVA, Kruskal–Wallis, Tukey HSD, and Mann-Whitney U tests. **Results:** A significant clinical appearance of the subjects' tongues was observed only between C and T1 ( $p=0.023$ ;  $p<0.05$ ). Significant hyphal formation was observed between C and T1

## RESUMO

Candidíase oral é uma infecção que ocorre na cavidade oral e é causada por espécies de *Candida*, frequentemente *Candida albicans*. Esta infecção geralmente ocorre em uma condição de imunossupressão causada por dexametasona. Devido aos efeitos colaterais de terapia antifúngica, o desenvolvimento de um padrão de modelo animal imunossuprimido para induzir candidíase oral para testar novas terapias se faz necessário. O objetivo deste estudo é observar candidíase oral em ratos Wistar imunossuprimidos pós-injeção de dexametasona utilizando doses de 7,2 mg / kg e 16 mg / kg. **Material e métodos:** Vinte e um ratos Wistar foram divididos em três grupos: grupo controle, tratamento grupo 1 (injetado com dexametasona na concentração de 7,2 mg / kg), e tratamento grupo 2 (concentração de 16 mg / kg) por cinco dias. A imunossupressão foi observada pela contagem de leucócitos e todos os palatos dos animais foram inoculados com *C.albicans* 0,1 ml de  $15 \times 10^8$  UFC / ml após 24 horas. As línguas dos animais foram observadas e confirmadas por exame laboratorial após 10 dias. A análise estatística foi realizada usando os testes de ANOVA um fator, kruskal – Wallis, Tukey HSD e de Mann-Whitney. **Resultados:** Significante diferença na aparência clínica da língua dos animais foi observada apenas entre C e T1 ( $p = 0,023$ ;  $p < 0,05$ ). Significativa diferença na formação de hifas foi observada entre C e T1 ( $p = 0,037$ ;  $p < 0,05$ ) e entre C e T2 ( $p = 0,007$ ;

( $p = 0.037$ ;  $p < 0.05$ ) and between C and T2 ( $p = 0.007$ ;  $p < 0.05$ ), and no significant difference was observed between T1 and T2. A significant increase in the colony count was also observed in similar results. **Conclusion:** Dexamethasone injection at doses of 7.2 mg/kg and 16 mg/kg is effective in triggering immunosuppression to induce oral candidiasis in immunosuppressed Wistar rats.

## KEYWORDS

Dexamethasone, immunosuppression, oral candidiasis.

$p < 0,05$ ), e nenhuma diferença significativa foi observada entre T1 e T2. Um aumento significativo na contagem de colônias foi também observado com resultados semelhantes. **Conclusão:** Injeção de dexametasona utilizando doses de 7,2 mg / kg e 16 mg / kg é eficaz no desencadeamento da imunossupressão para induzir candidíase oral em ratos Wistar imunossuprimidos.

## PALAVRAS-CHAVE

Dexametasona; Imunossupressão oral candidíase.

## INTRODUCTION

Oral candidiasis is the most prevalent opportunistic infection that occurs on the skin and mucous membranes of the oral cavity, and it is caused by candida species [1,2]. One of the opportunistic *Candida* that commonly causes the infections is *Candida albicans* (*C.albicans*), while a third of them are caused by non-albicans species [3] *C.albicans* is a harmless, normal flora found in the oral cavity of nearly 50% of the population, but in certain circumstances, such as in cases of decreased immune defenses or where there is a disruption of oral flora, *C.albicans* can turn into opportunistic pathogens [4].

Immunosuppression can increase the risk of candida infections. Immunosuppression is a condition where a person cannot respond to an infection normally due to a weakened immune system [5]. Some types of glucocorticoid drugs can cause immunosuppression, one of which is dexamethasone. Dexamethasone is often used in clinical practice, because it has adequate anti-inflammatory and immunosuppression effects, inhibiting the activation of T cell proliferation through increased activity of type 2 macrophages (Mph2), and reducing the B cell activator factor so that it causes autoreactive B cell apoptosis [6].

The innate and adaptive immune systems are responsible for maintaining *C.albicans* in a

commensal state. However, when the immune system is weakened due to immunosuppression, it fails to eliminate the adhesion of candida, which leads to oral candidiasis. A *C.albicans* infection triggers the differentiation of T helper (Th) cells to Th17 to produce Interleukin 17 (IL-17) through the activation of IL-23 produced by dendritic cells. IL-17A plays an important role in the mobilization and fungicidal activity of neutrophils. However, in immunosuppressed conditions, phagocytosis in polymorphonuclear cell disorders and macrophages decrease the quality and quantity of cytokines [7].

Several drugs that are used to treat oral candidiasis have side effects. Alongside the increasing number of cases of oral candidiasis, several studies have been performed to develop a new therapy that is more effective for treating oral candidiasis, but no studies have focused on developing a standardized immunosuppressed animal model to induce oral candidiasis for further research. Therefore, the authors conducted an experimental study about immunosuppressed Wistar rats that induced by dexamethasone. The dose of dexamethasone that are used were 7.2 mg/kg and 16 mg/kg, referring to previous studies [8,9]. In this study, it was expected that oral candidiasis can occur both clinically and microbiologically in immunosuppressed Wistar rats after inoculation of *C.albicans*. The aim of this experimental study was to observe oral

candidiasis in immunosuppressed Wistar rats due to dexamethasone injection at concentrations of 7.2 mg/kg and 16 mg/kg.

## MATERIALS AND METHODS

### Ethical approval

This study was approved by the Local Commission of Ethical Clearance (No. 323/HRECC.FODM/VI/2019). All procedures performed in the study were in accordance with the ethical standards of the institution which the study was conducted.

### Study groups

This research is an experimental laboratory with a post-test only control group design. The sample used was from 21 healthy male Wistar rats (*Rattus norvegicus*) weighing 160-250 grams, selected randomly into three groups (n=7): Wistar rats only inoculated with *C.albicans* (control group [C]), immunosuppressed Wistar rats induced with dexamethasone at a concentration of 7.2 mg/kg and inoculated with *C.albicans* (treatment group 1 [T1]), and immunosuppressed Wistar rats induced with 16 mg/kg dexamethasone and inoculated with *C.albicans* (treatment group 2 [T2]).

### Experimental Design

The immunosuppressed condition in rats was induced by dexamethasone (Mepro Farma Co., Jakarta, Indonesia) intramuscularly at 7.2 mg/kg and 16 mg/kg for five days. Blood films (via the tail vein: 0.10 ml) were collected on day six to measure immunosuppression through a leukocyte count. Rats were anesthetized using an intramuscular injection combining ketamine and xylazine (110 mg/kg and 8 mg/kg of body weight). On day six, immunosuppressed rats were then induced with *C.albicans* by inoculation of the palatal mucosa of all groups once, using a syringe with 0.1 ml Sabouraud Dextrose Broth (SDB), which contained  $15 \times 10^8$  UFC/ml *C.albicans*. The condition of oral candidiasis in rats was observed on day 10.

### Total White Blood Cell Counts

On day six, the blood (via the tail vein: 0.10 ml) of 21 rats per treatment was collected. Blood smears were made in an object-glass, were air dried then were fixed with methanol and stained with Giemsa's stain. Cell analysis was done under the light microscope by counting 100 cells, and the percentage of cells were calculated [10].

### Examination

#### *The Clinical Wistar Rat's Tongue*

Symptoms appearing on the tongue of the Wistar rats were observed on day 10, then the erythematous and/or pseudomembranous lesions score was determined (i.e., score (0) for samples that did not show lesions on the rats' tongues and score (1) for samples showing lesions).

#### *Unité Faisant Colonie (UFC) Counting*

The surface of the rats' tongues were swabbed twice with a cotton swab and the results were then put into the SDB liquid media to be diluted to  $10^{-4}$  and incubated for 24 hours. Next, the contents of the liquid media were taken using a micropipette of 0.05 ml and placed on Sabouraud Dextrose Agar (SDA), flattened using a spreader, and then incubated. The incubation process was performed anaerobically at 37°C for 48 hours then the fungal colonies were counted with the Loop tool [11].

#### *Gram Staining*

Fixation of *C.albicans* colonies to the surface of the microscope slide by heating. Then the slides were poured with a violet crystal dye and rinsed under running tap water to remove excess dye. Gram iodine mordant was applied for two minutes and briefly washed in tap water. The fasting was done by giving ethyl alcohol 96% for 5 seconds to 15 seconds, then immediately rinsed with water for one minute to stop the discoloration. Safranin was then dropped for 30 seconds and washed with water for a few seconds to finish the remnants of staining. The final stage was observed under a

light microscope with a magnification of 100x [12]. The results were then determined (i.e., score (0) for samples that did not show hyphal formation on the microscope and score (1) for samples that showed hyphal formation).

### Statistical Analysis

Data were analyzed using SPSS software version 10.05 (SPSS Inc., Chicago, USA). Data were expressed as mean  $\pm$  standard deviation (SD). The data was analyzed with the Kolmogorov–Smirnov test followed by Levene’s test ( $p>0.05$ ). The statistical analysis of the UFC counting and the number of leukocyte results was performed by a parametric test using a one way analysis variance (ANOVA) with Tukey’s HSD ( $p<0.05$ ). Meanwhile, a statistical analysis for the clinical examination and Gram staining results was performed by a non-parametric test using the Kruskal–Wallis test with the Mann–Whitney U test ( $p<0.05$ ).

## RESULTS

### Dexamethasone decreases the number of leukocytes in the Wistar rat

The Wistar rats’ leukocytes appeared purple under the microscope, spread between red blood cells, as shown in figure 1. The highest level of leukocytes was found in the control group, while the lowest was found in treatment group 2 (Figure 2). A normality test using the Kolmogorov–Smirnov test showed a normal distribution throughout the data ( $p>0.05$ ). Levene’s test results showed a homogenous data of the present study ( $p>0.05$ ). There were significant differences ( $p=0.00$ ;  $p<0.05$ ) in the one way ANOVA. The results of the Tukey HSD test stated that significance was observed between C and T1 ( $p=0.00$ ), C and T2 ( $p=0.00$ ), and T1 and T2 ( $p=0.00$ ) (Table I).

### The clinical appearance of rats’ tongues post inoculation of *C.albicans*

The observed clinical appearance of oral candidiasis on the rats’ tongues was in the form of pseudomembranous and/or erythematous type lesions. In the control group, in general,

there was no visible picture of oral candidiasis. This was in contrast to treatment group 1, where there was a visible picture of the erythematous type, with characteristics of mucosa atrophy and a red color due to increased vascularity. There was also the pseudomembranous type, which looks like a white pseudomembrane that can be scraped off. In treatment group 2, only the erythematous type of oral candidiasis was seen (Figure 3). The lowest median of lesions was found in the control group, while in treatment group 1 and treatment group 2 was observed in similar results (Figure 4). The Kruskal–Wallis test obtained significant results ( $p=0.039$ ;  $p<0.05$ ). The Mann–Whitney U test results stated that statistical significance was observed between C and T1 ( $p=0.023$ ;  $p<0.05$ ), while between C and T2 and between T1 and T2, no significant difference was observed ( $p>0.05$ ) (Table I).

### *C.albicans* colony count on UFC counting

The *C.albicans* colony looked white to beige, smooth, and circular with irregular edges, as shown in figure 5. The highest level of colonies was found in treatment group 2, while the lowest was found in the control group (Figure 6). The obtained data obtained was not homogeneous ( $p=0.017$ ;  $p<0.05$ ) and the Kruskal–Wallis test showed significant differences ( $p=0.01$ ;  $p<0.05$ ). The Tukey HSD test results stated that a statistical significance was observed between C and T1 ( $p=0.00$ ) and C and T2 ( $p=0.00$ ), while between T1 and T2 there was no significant difference ( $p=0.246$ ;  $p>0.05$ ) (Table I).

### The Results of Direct Mycology with Gram Staining

Under the microscope, in the control group, *C.albicans* cells were commonly seen as the formation of ovoid-shaped budding yeast cells, while in treatment group 1 and treatment group 2, in general, the cells were seen as hyphal cell shapes arranged in parallel form (Figure 7). The lowest median of hyphal formation was found in the control group, while in treatment group 1 and treatment group 2 was observed in similar results (Figure 8). The Kruskal–Wallis

test results showed a significant difference ( $p=0.009$ ;  $p<0.05$ ). The Mann–Whitney U test results showed that a statistical significance was observed between C and T1 ( $p=0.037$ ) and between C and T2 ( $p=0.007$ ), while between T1 and T2 there was no significant difference ( $p=0.317$ ;  $p>0.05$ ) (Table II).

**Table I** - Multiple comparisons of the Tukey HSD test results.

Variable	Group	C	T1	T2
Leukocytes Number	C	-	-	-
	T1	0.000*	-	-
	T2	0.000*	0.000*	-
Variable	Group	C	T1	T2
Colony Count	C	-	-	-
	T1	0.000*	-	-
	T2	0.000*	0.246	-

\*The result of multiple comparisons of the Tukey HSD test shows a significant difference between groups ( $p<0.05$ )

**Table II** - Multiple comparisons of the Mann–Whitney U test results.

Variable	Group	C	T1	T2
Score lesion on tongue	C	-	-	-
	T1	0.023*	-	-
	T2	0.107	0.317	-
Variable	Group	C	T1	T2
Score of Hyphal Formation	C	-	-	-
	T1	0.037*	-	-
	T2	0.007*	0.317	-

\*The result of multiple comparisons of the Mann–Whitney U test shows a significant difference between groups ( $p<0.05$ )

## DISCUSSION

Over the past few decades, the population of patients taking immunosuppressive drugs such as dexamethasone has gradually increased due to systemic illness. Therefore, the risk of oral candidiasis has also increased. Several antifungal agents, namely nystatin and azole, are reported to be resistant to oral candidiasis. Therefore, innovation to enhance the effectiveness of anti-fungal agents is needed [13]. This study developed an experimental

model of oral candidiasis in immunosuppressed Wistar rats. Induction using dexamethasone with inoculation of *C.albicans* was expected to suppress the immune system of the rat and create oral candidiasis. In this study, modifications were made to previous studies to provide an innovative and more effective model of oral candidiasis in immunosuppressed animals [8,9,14,15].

Dexamethasone is a glucocorticoid drug that has potent anti-inflammatory and immunosuppressant properties. The results of a data analysis on the number of leukocytes on the sixth day after a dexamethasone injection showed significant differences between groups. This shows that the injection of the dexamethasone drug is a proven indicator of the immunosuppressed condition in Wistar rats. A lower leukocytes number also indicates a weaker immune system because leukocytes play an important role in the innate and adaptive immune system. This is in accordance with previous research that explains that the injection of a single dose of dexamethasone in a rat can significantly reduce the number of leukocytes until day eight [16]. When the immune system declines due to the administration of dexamethasone, the body is unable to compensate for the infection such that the *C.albicans* cells turn into pathogens through several virulence factors [17].

In this study, the profile of oral candidiasis in Wistar rats was observed by a clinical examination of the rats' tongues and a laboratory examination (a UFC count and Gram staining). In general, in animal models of oral candidiasis research in acute conditions, pseudomembranous and/or erythematous lesions are clinically seen [18]. This is consistent with the results of research on clinical observations of Wistar rat tongues (i.e., pseudomembranous and/or erythematous lesions contained in the sample of treatment group 1 and treatment group 2). Based on a data analysis, showed that there were only significant differences between the control group and treatment group 1. In the saprophytic phase, *C.albicans* can also form

clinical lesions especially on the tongue, so there is a subject in the control group that shows the lesion. In one sample of treatment group 1 no lesions were seen. This is because the *C.albicans* in the rat's mouth failed to release antigens that could induce an inflammatory response. The diagnosis of one form of oral candidiasis can not only be made by clinical observation but needs to be confirmed by identification of *C.albicans* microscopically [19,20].

An accurate diagnosis of oral candidiasis can be confirmed by microbiological examination, that is UFC counting and Gram staining. In SDA, *C.albicans* cells can grow well, giving rise to a compact colony that is visible after 24 to 48 hours of incubation [20]. Yeast is the basis of their morphologic and biochemical characteristics. Useful morphologic characteristics include the size and shape of the cells, the color of the colonies, the presence of a capsule around the cells, and the production of hyphae or pseudohyphae [21]. The colony count examination results obtained significant results between the control group and treatment group 1 and also between the control group and treatment group 2. This shows that the number of colonies in treatment group 1 and treatment group 2 did not differ significantly.

On the Gram staining results, hyphal formation showed that the *C.albicans* was confirmed to be pathogenic. In acute candidiasis, fungal hyphae are seen penetrating the upper layers of the epithelium at acute angles. In addition, *C.albicans* cells in the form of pseudohypae are also found [22]. The results showed that there were significant differences between the control group and treatment group 1, and also between control group and treatment group 2. In the control group, there were two samples that looked like hyphae. This was probably due to *C.albicans* inducing immunosuppression through the release of TLR2-mediated IL-10 [23]. Besides, *C.albicans* can actively inhibit IL-17 production by altering the metabolism of tryptophan [24]. When rat immunity decreases, it is possible to form *C.albicans* hyphae. In addition to the control

group, failure to form hyphae in one sample of treatment group 1 showed that the sample had not experienced sufficient immunosuppression to convert *C.albicans* into pathogens. This can be influenced by internal and external factors, both from the rat's immune system and the surrounding environment.

The three oral candidiasis examinations, namely clinical examination, UFC counting, and Gram staining, generally showed significant differences between the control group and treatment group 1, as well as between the control group and treatment group 2, but there were no significant differences between treatment group 1 and treatment group 2.

## CONCLUSION

An intramuscular dexamethasone injection at a concentrations of 7.2 mg/kg and 16 mg/kg is effective in triggering immunosuppression in Wistar rats. Immunosuppression due to dexamethasone injection is effective in triggering oral candidiasis in wistar rats after inoculation of *C.albicans*.

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**Desiana Radithia**  
**(Corresponding address)**

Mailing address : Jl. Mayjen Prof. Dr. Moestopo No. 47, Surabaya, Indonesia 60132  
E-mail: deisy.radithia@fkg.unair.ac.id

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