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IL-17 plasma levels and erythrocyte sedimentation rate on oral candidiasis animal model

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ABSTRACT

Background: *A study with female animal models is important because the system immune of females is remarkably different from the male because of interaction between sex hormone and immune system. Interleukin-17 (IL-17) plays an important role in immune response toward Candida albicans (C. albicans) infection and Erythrocyte Sedimentation Rate (ERS) is an easy and sensitive test to assess the inflammation.* ***Purpose:*** *This study aimed to evaluate C. albicans infection, analyse the IL-17 levels and ESR in a female animal model of oral candidiasis.* ***Methods:*** *Female Wistar rats were used as oral candidiasis animal model. The rats divided into three groups (pre-treatment group (P0), 5th-day post-treatment group (P1) and 8th-day post- treatment group (P1). Each group consists of six rats. After the adaptation period, the P0 was sacrificed. The drinking water of the P1 and P2 was added tetracycline HCl 500mg /1L. On the day before and after inoculation C. albicans, Methylprednisolone was injected. Ten minutes before inoculation with 0.3 ml C. albicans 9.4 x 10^{7}/mL, rats were sedated by CPZ 0.7 mg IM. The rats in P1 group were sacrificed after five days and in P2 were sacrificed eight days after inoculation. The IL-17 plasma levels measured by enzyme-linked immunosorbent assay (ELISA), decreased on the 5th day but not on the 8th-day post-treatment. The obtained data were analyzed by parametric and non-parametric tests according to normality and homogeneity of the data with p<0.05.* ***Results:*** *The colony forming unit (CFU) of C. albicans collected over the mouth on increased almost 8-fold and on 8th-day post-treatment almost 3-fold compared with pre-treatment. The ESR increased on the 8th day but not on the 5th-day post-treatment.* ***Conclusion:*** *The IL-17 level was decreased on the 3rd day, ESR increased on eight days after inoculation of C. albicans in female rats' model of oral candidiasis.*

Keywords: *animal model; C. albicans; IL-17; infectious disease; medicine*

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INTRODUCTION

Candida albicans (C. albicans) is almost always found as a commensal microorganism in the healthy human oral cavity. The commensal properties of *C. albicans* are maintained by both innate and adaptive immune systems and normal flora bacteria *C. albicans* can grow commensals because *C. albicans* have the capability to avoid the immune system, called immune evasion. *C. albicans* can escape from the human immune system in various ways, including by regulating the complement cascade either by secreting enzymes secreting aspartyl proteases or by binding to the surface of complement regulators. During infection, complement facilitates the process of phagocytosis by opsonization and then initiates an inflammatory response by modifying the behavior of B cells and T cells.[1] *C. albicans* can also survive if phagocytized by leukocytes and can spread to other parts of the body (vomocytosis) and can cause leukocyte death.[2] If there is a change in the defense immune system of the oral cavity, it can trigger the shift of *C. albicans* from commensal into a pathogen.

The prevalence of oral candidiasis in infants is estimated at 5-7%, in acquired immunodeficiency syndrome (AIDS) patients 9-31%, and up to 20% in cancer patients. *C. albicans* is the cause of 75% candidiasis overall.[3] The incidence of human immunodeficiency virus (HIV) infection which

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caused AIDS, has increased very rapidly in this decade. The main complication of HIV infection is opportunistic infections including oral candidiasis.[4] According to study in Surabaya General Hospital, 2018, oral candidiasis was found in 57.14% of HIV patients.[5] The incidence of oral candidiasis was significantly correlated with the decrease of CD4 count in HIV patient.[6] The prevalence of auto inflammatory/autoimmune diseases due to therapy with immunosuppressant and cancer chemotherapy and radiotherapy also contribute for the enhancement of *C. albicans* opportunistic infections. *C. albicans* was isolated from 73.3% of patients who received chemotherapy in General Hospital Hasan Sadikin Bandung.[7] In the past two decades, it has been observed an abnormal overgrowth in the gastrointestinal, urinary and respiratory tracts, not only in immunocompromised patients, but also related to nosocomial infections and even in healthy individuals.[8] In addition to the oral cavity, *C. albicans* infections often occur in the vagina, gastrointestinal tract, skin and systemic infections called invasive candidiasis can also occur in the heart, eyes, intra-abdominal, joints, bones, and brain membranes.[9]

The immunocompromised patient needs long term antifungal drug as prophylaxis and therapy and this may lead to serious side effects and drug resistance. The prevalence of *C. albicans's* antifungal resistance is approximately 56.7% in patients with HIV infection.[10] This fact has resulted in the need for research to find alternative drugs that are safer for long-term use and this research requires appropriate animal models

Previous study believed the estrus cycle in females leads to significant variation response and need to increased sample sizes if using this gender in the study even though this is not proven. Female subjects are underrepresented in animal research across disciplines. The biological response, especially the immune response of women is different from that of men because of genes and, hormones can interfere with the immune response.[11] The unavailability of research using experimental animals causes a shortage of materials to be tested on women and prevents women from receiving treatment as well as men. On the other hand, various diseases caused by immune response deviations are more common in women.[12] In animal model, murine oral candidiasis, deviation of immune response was believed to have an important role in the pathogenesis of oral candidiasis.[13] In a previous study using the female murine model that mimics the natural infection in humans, the inoculation of C. *albicans* 2.5 x10^7 cells/ml (=10^6 cells/mouse) in prednisolone, tetracycline, and chlorpromazine treated caused high and stable colonization until seven days.[14]

In a study conducted by Sulistyani (2019), the injection of *C. albicans* cell wall intraperitoneally in healthy rat highly correlated with increase of IL-17 plasma levels.[15] IL-17 is the most potent inflammatory cytokine and most important cytokine toward *C. albicans* infection can eliminate *C. albicans* exposure rapidly.[16,17] These results also explain why, in healthy individuals, *C. albicans* cannot cause infection. The IL-17 induces adequate immune response to eliminate the *C. albicans*.

The evaluation of systemic inflammation in animal model of oral candidiasis has never been revealed. The hypothesis of this study was there were a reduction of IL-17 plasma level and an elevation ESR in the rat model of oral candidiasis. Thus, the purposes of the study are to analyze the plasma level of IL-17 and the erythrocyte sedimentation rate (ESR) in female Wistar rat (*Rattus novergicus*) as animal model of oral candidiasis. The ESR was chosen because it is an easy and sensitive marker of inflammation.

MATERIALS AND METHODS

The *C. albicans* ATCC 10231 was purchased from Biology Oral Laboratory, Faculty of Dentistry, Universitas Hang Tuah, Surabaya. Identification test was performed by culturing in the CHROMagar and the color of C. albicans colony was a green colony. CHROMagar technology is a color-based differentiation method. It is based on soluble colorless molecules (called chromogens), composed of a substrate (targeting a specific enzymatic activity) and a chromophore. When the target organism's enzyme cleaves the colorless chromogenic conjugate, the chromophore is released.[18] This strain was cultured in Sabouraud 4% dextrose agar (SDA) (Merck Cat. No 1.05438.0500) and then diluted in Sabouraud dextrose broth (Merck. Cat No 1.08339.0500) in concentration 9.4 x 10^7 cells/ml and stored at −4°C until the experiment was performed.

Six-week-old female Wistar rats (Iwan Farm, Pakis Aji, Malang) were used for all animal experiments. The rats were kept in cages with sufficient light from the sun, and the environmental temperature was constantly maintained at 26-29°C. One cage was used to keep three rats. Rats were given access to food and water *ad libitum.* Before the study began, it was confirmed that the female rats were not pregnant because they had been separated from the male rats since they were 15 day-olds. The two rats, which were randomly selected, were confirmed to be both in a healthy condition and not pregnant by a veterinarian at Veterinary Clinic, Livestock Office, Jember. All research procedures were approved by the Health Research Ethics Committee, Faculty of Dentistry, University of Jember with approval letter number 11280/UN25.7/KEPK/DL/2021.

The research design used was a pre-post separated group design. The experimental animals were divided into three groups, the Pre-Treatment Group (P0), the 5th Day Post-Treatment Group (P1), and the 8th Day Post-Treatment Group (P2). After adaptation for 10 days, rats in the P0 group were sacrificed and intracardiac blood was taken for the measurement of the variables. The drinking water of the rats in the P1 and P2 groups were given Tetracycline HCL (Sanbe Pharmacy, Indonesia) in a concentration of 500 mg/L to reduce the number of oral floras, which have capability to inhibit *C. albicans* growth. Tetracyclines are

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broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms.

On the day before treatment, the rats in the treatment (P1 and P2) groups were injected subcutaneous with Methylprednisolone sodium succinate (MP) (Phapros Ltd, Indonesia) in dose 40 mg/kg.BW. On the treatment day, the rats were sedated with Chlorpromazine (Phapros Ltd, Indonesia) injection 5 mg/kg.BW IM. Chlorpromazine is a psychotropic agent indicated for the treatment of schizophrenia. It also exerts sedative and antiemetic activity. After rats were sedated, 0.3 ml suspension of *C. albicans* ATCC 10231 in concentration 9.4 x 10^7 cells/ml (3.1 x 10^6 cell/rat) was inoculated in the oral cavity of the rats with a small plastic brush stick**.** On a day after inoculation, rats were injected with MP again in the same dose as before treatment. On the 5th day of treatment, the rats in the P1 group were sacrificed and on the 8th day the rats in the P2 group were sacrificed. The sacrifice procedure of rats followed the rules of Animal Euthanasia Policy of University in St Louis USA. The rats were anesthetized with Ketamine-Xylazine cocktail (0.1 ml Ketamine 1000mg/10mL+ 0.05ml Xylazine 20mg/50mL) and followed by exsanguination.[19,20] All procedures are shown in Table 1.

The infection of *C. albicans* was determined by microbial evaluation: the whole oral cavity, including the buccal mucosa, tongue, soft palate, and other oral mucosal surfaces, was swabbed using a sterile cotton swab then, the end of the cotton swab was cut off and placed in a tube containing 5 ml sterile saline. To dissolve *C. albicans* from the swab into the saline, we used a Vortex. Then, after serial 100-fold dilutions, the cell suspension was incubated on Sabouraud dextrose agar + Chloramphenicol (Condalab Cat. No 1134, Madrid Spain) at 37°C for 20 hr. The colony forming units (CFUs) of Candida colonies were counted**.** The number of CFUs was used as the marker of *C. albicans* infection.

The ESR was measured using an ESR fast detector, and 1.25 ml ESR vacuum tubes containing sodium citrate (Monotes™, Zhejiang, China). ESR was performed immediately after blood had been drawn to avoid changes in the blood composition due to storage. The blood was put in an ESR tube, until the line mark and shaken 90 degrees six times so that the blood was well-mixed. After that, the tube was placed in an ESR Fast-Rack ESR rack. The ESR rack was confirmed flat by adjusting the mark in the rack. The timer was turned on when the tube had been inserted on the rack. The ESR was observed after 30 minutes.

The level of IL-17 was measured using IL-17 Rat Elisa Kit (MyBioSource catalog No. MBS164772 R, Sandiego, California USA) The blood was inserted into a 3 ml vacutainer containing ethelene diamine tetra-acetic acid (EDTA) (Vaculab™, with lilac color cap Dubai United Arab Emirates) then the plasma was separated using a mini centrifuge. The plasma separation was performed immediately after collection to avoid blood lysis. The obtained plasma was inserted into the mini tubes and stored in a styrofoam box containing ice gel, and the box was kept in a freezer at -4°C until brought to Surabaya for analysis. Elisa IL-17 analysis was carried out at the Laboratory of Specialised Hospital for Infection, Universitas Airlangga.

The data were analyzed using Statistical Package for Social Science (SPSS) version 22 (IBM®, SPSS®, Illinois, Chicago, US). To analyze the distribution and homogeneity of data, we use Shapiro-Wilk and Levene tests. If the data were normally distributed and homogeneous, the differences between groups were analyzed using a one-way analysis of variance (ANOVA) test followed by a post-hoc least significant difference (LSD) test to compare differences between all groups. If the data were not normally distributed neither homogeneous, the differences between groups were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney test when the data were not normally distributed or/and non-homogeneously distributed, the p value was set at <0.05.

Table 1. The treatment of animal model of oral candidiasis

Day	-2	-1	0	1	2	3	4	5	6	7	8
PO	sacrificed										
P1	given drinking water + tetracycline	Injected with MP	Injected with CPZ + inoculation C. albicans	Injected with MP				Sacrificed			
P2											Sacrificed

Table 2. The mean and standard deviation of CFU number, IL-17 level, and ESR in the pre-treatment, 5th day post-treatment and 8th day post-treatment groups

Variable	Mean ±SD		
	Pre-treatment	5th day post-treatment	8th day post- treatment
CFU (x 10^5)	0.125 ± 0.049	9.778 ± 4.259	3.057 ± 0.963
IL-17 level (pg./L)	79.98 ± 4.96	55. 62 ± 6.03	52.56 ± 9.97
ESR/hour	4.5 ± 0.55	4.83 ± 0.75	5.83 ± 1.17

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RESULTS

The means and standard deviations of CFU in P0, P1, and P2 are shown in Table 2. The CFU on the 5th-day post-treatment group increased almost 8-fold compared with the pre-treatment group. On the 8th-day after treatment, the CFU tended to decrease but was still nearly 3- fold compared with the pre-treatment group. The distribution and homogeneity test data using Shapiro-Wilk and Levene test showed that the data were in normal distribution and homogenous. The mean differences between groups were analyzed by a one-way ANOVA test, followed by LSD. The result showed that the mean of CFU *C. albicans* increased in the P1 and declined in the P2 but still more than the P0 group.

The test of normality and homogeneity data results showed that the IL-17 level data were neither distributed normally nor homogeny. The mean differences of IL-17 plasma level analysis between groups were used with a non-parametric test, namely the Kruskal-Wallis test followed by the post hoc Mann-Whitney test. The result showed that the levels of IL-17 between the pre-treatment and 5th-day post-treatment groups were significantly different. The IL-17 levels between the P1 and the P2 were not different significantly ($p<0.05$). The IL-17 levels between the P0 and the P2 group were not different either. From these facts, it can be concluded that the levels of IL-17 in female animal Wistar rat models of oral candidiasis decreased starting on the 5th day post treatment (P1) and persisted until 8th day post treatment. This fact indicates that the decrease in IL-17 levels occurs in animal models of oral candidiasis.

The normality and homogeneity ESR data test were also neither normally distributed nor showed homogeny. The analysis of the difference test was using the same test as IL-17. The results showed a difference between groups P0 and P2 ($p<0.05$), but not between P0 and P1. This result indicates an elevation of ESR in the experimental animal model of oral candidiasis of female Wistar rats on the 8th post-treatment group but not on the 5th day post-treatment group.

DISCUSSION

The number of *C. albicans* colonies from the entire oral cavity on 5th day after inoculation of *C. albicans* increased almost 8-fold. On 8th day after exposure, the number decreased but was still three times higher than the pre-treatment group. This fact indicates that a very significant infection occurred. In five days, 3.1×10^6 cell (spores) of *C. albicans* had become 9.7×10^5 CFU. In studies using healthy mice exposed to the highly infectious strain of *C. albicans* SC5314 or its derivatives, the immune response was showed very rapidly and colonization of C. albicans did not occur. Effective activation of the immune response toward *C. albicans* leads to the rapid elimination of *C. albicans* only in a few days.[21] Therefore, in producing a model of *C. albicans* infection, it is necessary to reduce the immune system, and reduce local oral defense factors, which is in this study were done by injection of MP and giving a broad-spectrum antibiotic Tetracycline HCL, in drinking water.

The result showed that the level of IL-17 reduced on 5th day post treatment. This decrease was probably due to the injection of Methylprednisolone sodium succinate. Methylprednisolone Sodium Succinate is the sodium succinate salt of a synthetic glucocorticoid receptor agonist with immunosuppressive and anti-inflammatory effects. Methylprednisolone sodium succinate is converted into active prednisolone in the body, that diffuses passively across cell membranes and binds to intracellular glucocorticoid receptors. This complex translocates into the nucleus, where it interacts with specific DNA sequences, resulting in increased or suppressed transcription of certain genes. The methylprednisolone-glucocorticoid receptor complex binds to and blocks the promoter sites of proinflammatory genes, promotes the expression of anti-inflammatory gene products, and inhibits the synthesis of inflammatory cytokines, primarily by blocking the function of transcription factors, such as nuclear factor-kappa-B (NF-kB).[22]

Considerable evidence in both humans and mice reveals a clear and specific role for IL-17 in protection against the *C. albicans.* [21,23] The natural resistance of mice to *C. albicans* is highly dependent on the functional IL-17 pathway.[21] The T-helper (Th)-17 response during disseminated fungal infection can be both protective and detrimental. Splenocytes isolated from hIL-37Tg mice with a higher fungal burden produced significantly more IL-17 in response to *C. albicans* pseudohyphae.[24] The immune response to *C. albicans* is initiated by the binds of its Pathogen Associated Molecular Pattern (PAMPs) with the Pattern Recognition Receptors (PRR) of immune cells. This binding induces intracellular signaling cascades, then triggers the secretion of various inflammatory cytokines. Some interleukins induce differentiation of naive Th0 to Th17 through the enhancements of Signal Transducer and Activator Transcription 3 (STAT3) and Retinoid-Related Orphan Receptor γt (RORγt). Th17 will produce the IL-17 that trigger epithelial and mesenchymal cells to express chemokines for recruitment of neutrophils (Interleukin-8, C-X-C motif ligand-1 (CXCL1), CXCL5), Granulocyte-Colony Stimulating Factor, and antimicrobial peptides (AMP) such as defensins and protein S100.[25] Those chemokine trigger immune cells, particularly neutrophils, move to the site of infection and induce secretion various other inflammation mediators.[26,27] Excessive IL-17 production can trigger various autoimmune and autoinflammatory diseases.[15] On the other hand, deficiency in the IL-17 pathway can lead to bacterial and fungal infections and promote tumor growth. IL-17 deficiency states are associated with susceptibility to infections from *C. albicans*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*. [28]

Examination of the ESR is an easy and sensitive examination of changes in the body, particularly

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inflammation condition. In this study, the ESR increased on 8th day post-treatment and not yet on the 5thday post-treatment. The increase in ESR was mainly due to an increasing level of inflammation-sensitive plasma proteins (ISPs), a plasma protein that eliminates infection. The plasma proteins usually have a positive electrical static charge. Red blood cells in healthy conditions have negatively charged, so they repel each other. The positive electrical static charge of plasma proteins can neutralize the negative charge of red blood cells so that red blood cells easily attach each other and form rouleaux. In the rouleaux form, the erythrocytes will settle faster. than single red blood cells.[29] In 8th day post-treatment, the ESR increased indicated that inflammation-sensitive plasma proteins (ISPs) were elevated in blood. ISPs can reduce the infection. This result was in accordance with the decreased the number of CFU of *C. albicans*. On the 8th day post-treatment group, the inflammation elevated and caused a decrease in the number of *C. albicans* CFU.

The inflammatory response toward various conditions including fungal infection leads to a significant alteration in the plasma level of several proteins. The measurement of the plasma protein level is applied to determine the normal/abnormal response of the host to tissue injury and some extent, indicate the amount of tissue involved. The inflammatory response leads to a decrease of albumin and transferrin levels, and enhancement of haptoglobin level as a response to blood loss. The level of coagulation factor for example fibrinogen, globulins, and protease inhibitors, observed elevation from 10% until 5-fold the baseline level by the cause of injury and at the stage of the process when the sample was collected.[11] Several plasma proteins that increase during inflammation are called inflammation-sensitive plasma proteins (ISPs), including fibrinogen, haptoglobin, 1-antitrypsin, serum amyloid A, C-reactive protein, and orosomucoid. [12,13]

This study has several limitations such as the separated pre-post design could not accurately describe the pre-post condition, but the study design was chosen because 5 mL of blood to measure all dependent variables is needed. It should be understood that this study was only basic research that provides a new perspective that female laboratory animals can be used as an experimental model. A very severe infection may develop in female laboratory animals receiving the treatment we gave. In conclusion, there was significant infection even up to the 8th day after exposure; the IL-17 reduced on both the 5th and the 8th day after treatment. The ESR increased on the 8th day after treatment in our oral candidiasis model. Further study could use the oral candidiasis animal model for the treatment exploration of oral candidiasis with various methods.

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