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Expression Levels of Intestinal Toll-Like Receptors 2 and 4 in Acute Inflammation Caused by Intestinal Candidiasis: An Experimental Study in Wistar Rats

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Abstract

Background: *Candida albicans* grows in the gastrointestinal tract as a normal microflora that may cause intestinal candidiasis, characterized by formation of biofilm and inflammation. *Candida* is recognized by toll-like receptors (TLRs). This study aimed to explore the relationship between intestinal TLR 2 and TLR 4 expression levels in candidiasis at each phase of inflammation.

Methods: An experimental study was performed using a simple randomized sampling on 30 male Wistar rats divided into a control and a treatment group. Each group was inoculated with Candida albicans. Dysbiosis conditions were designed in the treatment group using multiple antibiotics and on day 5 the rats were injected with subcutaneous cortisone acetate. The groups were terminated in five different times (days 7, 14, 21, 28 and 35). On the termination day, intestinal tissue was isolated and the TLR 2 and TLR 4 expression were analyzed by immunohistochemistry. The data were analyzed by parametric test with SPSS ($p<0.05$) and completed by post-hoc test Least Significance Difference (LSD) to compare pairs of groups.

Results: The expression of TLR 2 and TLR 4 between control and treatment groups showed significant differences ($p=0.005$). In the treatment group, there was a gradual increase in the TLR2 and TLR 4 expressions. Positive expression of TLR appeared more in the submucosal or basal area than apical surface. The treatment group showed the highest expression of both TLR2 (82.37%) and TLR4 (87.40%) on termination day 35.

Conclusions: Inflammation caused by intestinal candidiasis can result in increased expression of intestinal TLR2 and 4 contributing to an increased risk of biofilm formation.

Keywords: *Candida albicans,* candidiasis, inflammation, intestinal, TLR2, TLR4

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Introduction

Various types of microorganisms such as bacteria and fungi, known as normal microflora grows naturally in human digestive tract. The use of antibiotics orally shows long-term effects on the balance of gut microflora and composition of microbiota that can lead to dysbiosis.[1] Candidemia or candidiasis often occurs due to nosocomial infections, followed by several risk factors such as prolonged hospitalization, antibiotic treatment, and catheterization. Patients at risk who are exposed to antibacterial agents will be more susceptible to *Candida spp*.[2]

Each year, more than 3 million people are

affected by life-threatening invasive fungal infections. Fungi such as *Candida albicans* are commensal organisms that cause disease when the host immune balance is disturbed.[3] More than 90% of invasive candidiasis comes from infections with *Candida albicans, Candida tropicalis, Candida parapsilosis, Candida glabrate,* and *Candida krusei*. *Candida albicans* is the largest and most dominant fungal pathogen.[4] Approximately 75% of women will experience a fungal infection at least once in their lifetime, furthermore fungal infections can cause infections that spread through the bloodstream with a mortality rate of around 47% in some cases. *Candida albicans* infections cause 15% of sepsis and 40% of bloodstream infections.[5]

Candida albicans grows in the gastrointestinal tract as normal microflora and causes intestinal candidiasis under certain circumstances such as use of long-term antibiotic. Dysbiosis due to overexpressed of fungal could change the composition of microbiota and lead to imbalanced immunity.[6] In normal circumstances of the mice's gut, *Candida albicans* does not colonize. Infection of *Candida albicans* may be asymptomatic and symptoms could manifest in a disruption of the homeostasis state of the host.[7] Proteins expressed especially in *Candida albicans* cell walls, namely polysaccharides, are recognized by the immune system and have the ability to activate both toll-like receptor (TLR) 2 and TLR 4 followed by cytokine activation and trigger inflammation.[8] The virulence and resistance of Candida albicans are determined by its ability to form biofilms through complex mechanisms.

The immune system has the ability to recognize *Candida* through innate or adaptive immunity. The innate immunity response is the first defense system against pathogens and plays an important role in regulating the burden of fungi and preventing disease. Recognition of *Candida* in innate immunity through the identification of molecules pathogen-associated molecular patterns (PAMPs) in *Candida*. These PAMPs will be recognized by the host immune system via receptors called pattern recognition receptors (PRRs).[9]

The recognition process of *Candida* cell wall by TLR, leads to activation of the innate immune response and restricts the rapid propagation of fungi. Attachment and interaction of fungi with multiple PRRs increase immune response to eradicate pathogens.[10] Co-engagement of TLR and antigenic protein of fungi, induces excessive inflammation.[11] The TLR is an important PRR and is involved in the recognition of PAMPs in microorganisms. This receptors are type I transmembrane proteins that have an important key in regulating the immune system against microbial infections.[12]

The TLR 4 receptor binds myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) leading to activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs), thus generating production of proinflammatory cytokines and chemokines. The TLR2 also primarily recognizes cell wall of fungi, through MyD88 activating NF-κB and MAPKs.[10] In individual with immunosupressive condition, infection of *Candida* contributes to biofilm production. Biofilm is the evasion mechanism of *Candida* against the immune response. It is important to investigate the expression of TLR and its association with inflammation responses based on stage in candidiasis. Biofilm formation is a phase when candida infection is in a stage that is difficult to treat. Candidiasis in the biofilm phase influences TLR signaling and indicates host-microbe interactions, so that the role of TLRs in the disease status and stages of inflammation is well understood. This study aimed to describe the association of TLR 2 and 4 expression in a rat candidiasis model with the inflammation phase that may contribute to biofilm formation.

Methods

This study was an experimental case-control laboratory study conducted in the Embryology Laboratory of Veterinary Faculty, Airlangga University and Bioscience Laboratory Brawijaya University using a simple randomized sampling method. As many as 30 candidiasis rats model from male Wistar rats (*Rattus norvegicus*) was included and divided into 2 groups, namely the control group and the treatment (case) group. Each group was further divided into 5 groups with different termination times.

Dysbiosis conditions were designed in the treatment group by using multiple antibiotics tetracycline (25 mg/ml), streptomycin (20 mg/kg), and gentamycin (7.5 mg/kg) orally on days 1, 2, 3, 4, and 5. On the 5th day, rats were injected with subcutaneous cortisone acetate (225 mg/kg).[13] Candida infection in rats occurred on day 6, with oral inoculation of 0.1 ml of *Candida albicans* inoculum (1x106 *Candida albicans*). On day 7 until termination

time, the rats were given Spider medium twice a day (2.5 ml).[14] The control group was given aquadest orally. Termination was conducted at 5 different time, namely days 7, 14, 21, 28, and 35 after inoculation of *Candida albicans*.

Immunohistochemical analysis of TLR2 and 4 from the intestine (*caecum*) was observed using a light microscope at 400x magnification to visualize the TLR 2 and 4 expressions (Scytek AMF 080), that was observed in brown cells. The TLR expression in cell was quantified with the ImmunoRatio analysis tools and calculated as the percentage of TLRs expressed in cells.

Caecum tissue sections 5 µm thick were incubated with primary antibodies (TLR2 1:100, rabbit polygonal Santa Cruz Biotechnology; TLR4 1:100, monoclonal IgG mouse, Santa Cruz Biotechnology) for 1.5 hours. The sections were incubated with Ultra Tek anti-polivalent, then incubated with Ultra Tech HRP. Subsequently, the sections were stained with Chromagen DAB counterstained with hematoxylin and covered with coverslip. Cells that expressed TLR 2 and 4 would turn dark brown under a microscope.

Data for morphometry and quantitative staining were analysed immediately after the staining procedure using a 400x microscope in two certain fields. The expression of TLR 2 and 4 were observed in colored brown cells. Furthermore, the TLR 2 and 4 expression were quantified by calculating the TLRs expression as a percentage using the ImmunoRatio analysis tools. The percentage of positively stained nuclear area, designated as a labeling index, was stained using a color deconvolution algorithm, for separating the staining components (diaminobenzidine and hematoxylin) and adaptive thresholding for nuclear area segmentation.[15] The percentage of expression referred to the positively stained (brown) nuclear of all cells in a certain area in one microscope field .

The data of TLR2 and TLR4 in the control and treatment groups were analyzed by parametric test with SPSS ($p<0.05$) and completed by the least significance difference (LSD) post-hoc test to compare pairs of groups for statistical analysis. This study has received approval from the Ethics Committee of the Faculty of Medicine, Wijaya Kusuma Surabaya University (No. 10185/SLE/FK/UWKS/2016).

Results

The TLR in cell showed positive expression, observed by brown nuclear staining. The mean percentage of TLR expression in each group was depicted in Table 1.

In the control group, the mean percentage of TLR 2 and 4 did not differ in various termination days ($p=0.870$ and 0.458, respectively). Interestingly, the expression of TLR 2 and 4 in intestine was significantly different between time of termination ($p=0.001$ and 0.000, respectively). Moreover, the highest expressions of both TLR 2 and 4 at termination day 35 was 82.37% and 87.40% as depicted in Table 1.

The expression of TLR 2 and 4 increased significantly in the treatment groups, especially on days 21, 28, and 35. Expression of TLR 2 on termination day 7 did not show any difference with day 14 of termination. The percentage of TLR 2 expression on day 35 showed the highest among the others, but did not differ significantly with days 14 and 18, and a similar tendency was also observed in TLR 4 expression (Figure 1).

The expression of TLR 2 between control and treatment groups showed differences especially in TLR 4 expression. This was in line with the results of TLR 4 percentage and immunohistochemistry, and interpreted by

Table 1 Mean Percentage Changes in TLR 2 and TLR 4 Expression According to Termination Time in Intestine (*Caecum*) Rats

Termination Day	TLR 2 (%)		P-value	TLR 4 (%)		P-value
	Control Group	Treatment Group		Control Group	Treatment Group	
Day 7	48.58	49.10	**0.086****	**47.90**	**44.68**	**0.005****
Day 14	54.35	55.98		**53.05**	**48.72**	
Day 21	50.45	71.70		54.86	62.37	
Day 28	50.15	67.78		56.12	64.03	
Day 35	44.30	82.37		46.02	87.40	
P-value	0.870*	0.001*		0.458*	0.000*	

Note: *ANOVA test between group, *Independent T test between control and treatment groups

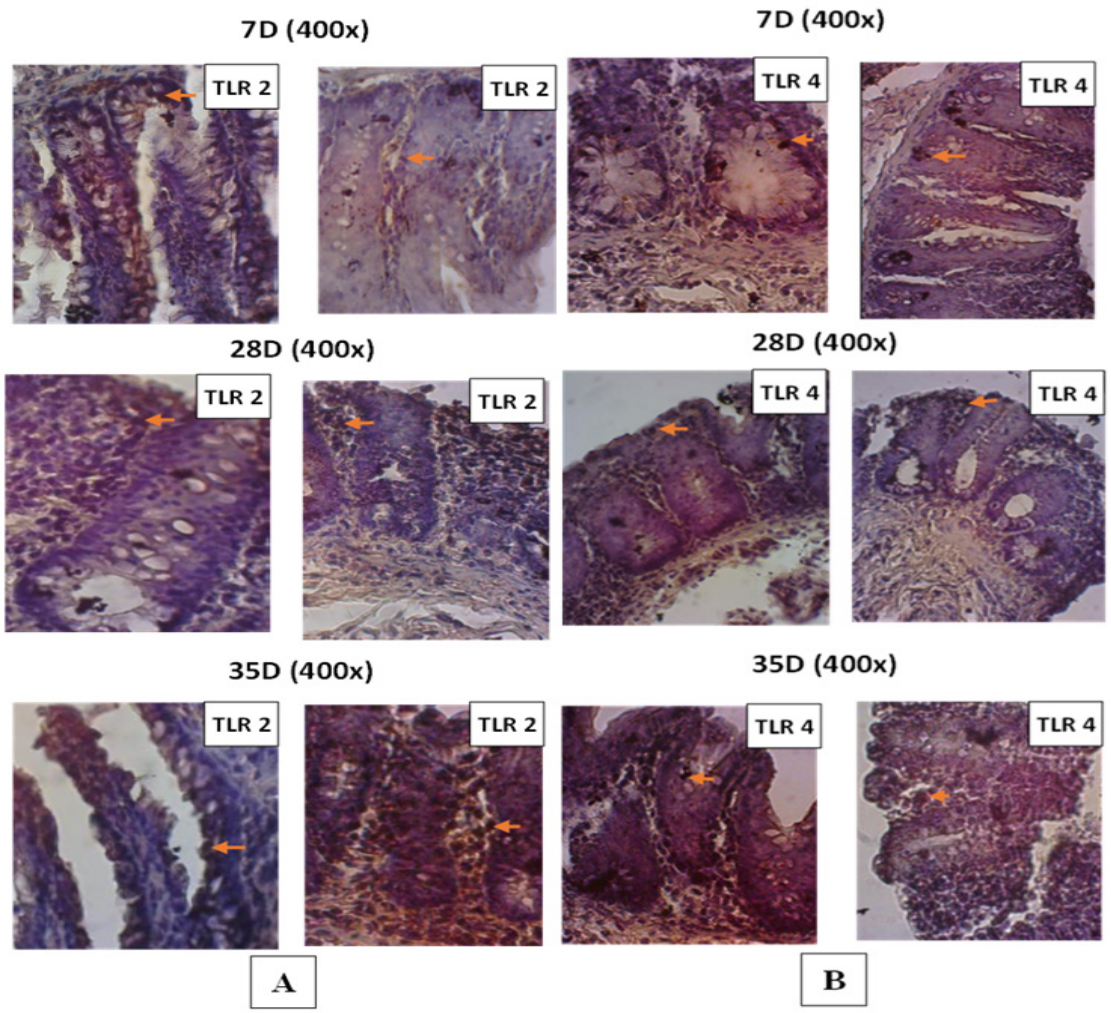

Figure 1 Expression of TLR2 (A) and TLR 4 (B) in the Intestine of Rat (400x Magnification) in the Control Group (Left Pane) and Treatment Groups (Right Pane) Observed on Days 7, 28, 35. Arrows show the TLR Expression in cells.

statistical analysis (p=0.005). Expressions of intestinal TLR 2 and 4 were evaluated by immunohistochemistry method. Expression of TLRs was indicated by the presence of brownish granules (Figure 1). Between control and treatment groups in TLR4 expression showed statistical differences (p=0.005). Both TLR 2 and 4 were strongly expressed in intestinal epithelial cells in crypts, the villous-crypt junction and lamina propria. Positive expression of TLR appeared more in the submucosal area than the apical surface. The percentage expression of TLRs increased at the time of termination and the highest expression of TLR 2 and 4 was observed at termination day 35. In the treatment group, a gradual increase was observed in the expression of TLR 2 and 4.

Discussion

Candida albicans has virulence factors, which cause candidiasis and contribute to the development of biofilms. The microenvironment consisting of biofilms make them more resistant to eradication and is accompanied by antimicrobial insensitivity. The main findings of this study are that infection of *Candida albicans* leads to candidiasis in the intestine, expression of TLRs signals the onset of inflammation associated with fungal infiltration and biofilm formation. The TLRs are part of the innate immune system that recognizes molecules patterns associated with pathogens activated by similar ligands. Activation of TLR stimulates

the host inflammatory response to protect against infection.[16] Both TLR 2 and 4 showed lower expressions in the control group than in treatment group.

On days 7 and 14, expression of TLR 2 and 4 increased slightly in the treatment groups, probably because of the initial inflammation response after inoculation of *Candida albicans* attached to the intestinal mucosa. This is likely because the presence of β-glucan in the cell walls of *Candida albicans* will stimulate the TLR 2 and 4 expression in the rat intestinal mucosa. This β-glucan acts as PAMPs that is first recognized by TLR. The process of recognizing β-glucan in *Candida albicans* by TLR 2 subsequently induces the formation of pro-inflammatory cytokines.[17] Activation of the immune system by TLR will further activate a number of pathways, such as myeloid differentiation primary response protein (MyD88). Furthermore, MyD88 will stimulate the translocation of NFκB into the nucleus and lead to the production of pro-inflammatory cytokines, TNFα and IL-6.[18]

Positive expression (brownish granule cells) is in low percentage at early stage of candida infection such as days 7 and 14. This shows that only a few colonies of Candida albicans cells attached to the intestinal mucosa in the treatment and control groups, which had the same number of *Candida albicans* as normal microflora. On day 21 after inoculation, *Candida albicans* colonized and proliferated in the gastrointestinal mucosa of the treatment group. Intestinal histopathological changes represented increased TLR expression over time. The number of *Candida albicans* cells (CFU) increased and experienced overgrowth compared to day 7 after inoculation.[19] At this stage, the expression of TLR 2 and 4 began to increase. Furthermore, on the days 28 and 35 after inoculation, TLRs expression increased significantly and its intensity was very strong. In addition, activation of the immune response occurs chronically, inducing systemic inflammation. Inflammatory involvement in candidiasis is a double-edged sword. For this reason, TLR signaling should be closely monitored to determine the causes of dysfunction of the immune response and inflammation. Chronic inflammation influences biofilm formation.[20] A study of oral mucosal epithelial cells toward leptospiral infection in Thailand described that leptospirosis in fatal cases showed increased levels of inflammatory cytokines mediated by activation of hTLR2.[16]

Biofilm is a group of highly structured colonies arranged in such a way that they are covered by an extracellular polymer matrix.[21] The main components of mature biofilm are carbohydrates consisting of β-1,6-glucans and α-mannan polysaccharides.[22] On day 35, the β-glucan content increased so that it could stimulate the TLR 2 and 4 expression much higher than the previous termination days, namely days 7, 14, and 21.

In the following phase, for example day 35, TLR 2 and 4 expression were more dominant in the submucosal area. In the submucosal area or basal surface, biofilms allow for easier absorption of nutrients. Biofilm composed of many types of microbe and produce extracellular matrix such as β-glucans. Study from the University of Connecticut to characterize biofilm on oral mucosal described that β-glucans from biofilm was shown in basal area. At the biofilm form there is a mechanism for candida to evade the immune system so it arranged hidden in the basal area. TLR is a receptor that recognize the foreign material such as β-glucans from biofilm found in submucosal or basal of intestine.[23] Surface epithelium is responsible as front liner of the innate system in mucosal digestive tract.[24] This receptor acts in important role of microbiota intestinal which considered to be intermediaries between the intestinal epithelial barrier, microbiota and immune.[25]

Analysis of TLR expression depends on color staining in immunohistochemistry methods. The difference in color analysis subjectivity limits this study, therefore, control group especially in incubation time is very crucial.

In conclusion, inflammation caused by intestinal candidiasis in rats likely results in increased expression of intestinal TLR 2 and 4. Blocking of TLRs leads to impaired of immune response to *Candida albicans*. The TLR 2 and 4 are the pathways and mechanisms of the immune response to *Candida albicans* infection and intestinal inflammation. Along with the occurrence of chronic inflammation, the risk of biofilm formation may occur. Therefore, elevation of TLR 2 and 4 expression will be an indicator of biofilm formation in the intestine.

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