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Comparison Study of Phagocytosis Activity among Live *Candida albicans*, Live and Heat-treated *Saccharomyces cerevisiae*

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Abstract

Fungi and yeast can stimulate neutrophil activity through phagocytosis mechanism that can cause inflammation and pathogenesis. The activation of neutrophil phagocytosis by baker's yeast that was heated at baking temperature has not yet been clearly defined. This study aimed to compare the phagocytosis activity among live *Candida albicans* (CL), live *Saccharomyces cerevisiae* (SL) and *Saccharomyces cerevisiae* that was heated at baking temperature (SH). Phagocytosis activity was assessed by mixing whole blood from 30 healthy volunteers with normal white blood cells and absolute neutrophil counts with a yeast suspension adjusted to an absorbance of 600 nm at 5. The phagocytosis percentage and phagocytosis indexes were determined at 0, 5, 10, 15, 20, 25 and 30 minutes of incubation. At 25 minutes of incubation, it was found that the median [interquartile range] of phagocytosis percentage against CL, SL and SH were 90.0 [85.0-97.0] %, 84.0 [78.0-91.0]% and 43.0 [28.0-74.5]%, respectively. The median [interquartile range] of phagocytosis index against CL, SL and SH were 2.62 [1.83-3.26], 1.90 [1.46-2.14] and 0.57 [0.40-1.48], respectively. The phagocytosis percentage and index between CL versus SL, CL versus SH and SL versus SH were significantly difference (p-value < 0.05). This suggested that *Saccharomyces cerevisiae* with heat-treated at baking temperature was still capable to stimulate phagocytosis, although the phagocytosis percentage and phagocytosis index of both of these live yeast cells were significantly higher.

Keywords**:** *Candida albicans; Saccharomyces cerevisiae; neutrophil; phagocytosis; inflammation.*

1. Introduction

Body clearance of fungi, *Candida albicans* and *Saccharomyces cerevisiae* involves phagocytosis by fixed tissue macrophages, infiltrating monocytes and neutrophils by using both the oxidative and non-oxidative killing (Small et al., 2019). Neutrophils are the first responders in acute inflammation. Recent studies show that neutrophils contribute in pathology of chronic inflammation with various mechanisms, however the role of neutrophils in chronic inflammation show several common mechanisms, for examples, serine protease releasing, the formation of neutrophil extracellular traps (NETs) and the activation of other immune cells. Although in chronic inflammation, the role of neutrophils is less well understood than mononuclear phagocytes, they also play a role in humans with chronic inflammatory diseases such as psoriasis (Herrero-Cervera et al.,

2022). In psoriasis, many triggers can activate immune response in loop and lead to the assembling of neutrophils that generate hyperproliferative keratinocytes in the epidermis region (Mohd-Noor et al., 2022). The fungal colonization is implicated in the pathogenesis of psoriasis. From meta-analysis, the detection rates of Candida species for psoriatic cases were significantly higher than for healthy controls (Pietrzak et al., 2018). *S. cerevisiae* is generally inoculated into bread dough at a concentration of 2% of the total ingredients, and the yeast multiplies, leading to an increase in the volume of the bread dough due to the production of fermentation gases (Parapouli et al., 2020). Although all yeast cells may die at the general temperature for baking bread (175 to 190 °C), the capability of the heat-treated yeast cells for activation of neutrophil phagocytosis is not clearly defined. Therefore, the study of capability of heat-treated *Saccharomyces cerevisiae* for activation of neutrophil phagocytosis will be an evidence for reconsideration of the role of heat-treated yeast cells as phagocytosis trigger. In this study, attempts were made to determine and compare the phagocytosis activity of neutrophils using live *Candida albicans* (CL), live *Saccharomyces cerevisiae* (SL) and *Saccharomyces cerevisiae* that was heated at baking temperature (SH) as triggers.

2. Objectives

This study aimed to study and compare the phagocytosis activity of neutrophils obtained from 30 healthy volunteers against live *Candida albicans* (CL), live *Saccharomyces cerevisiae* (SL) and heat-treated *Saccharomyces cerevisiae* (SH).

3. Materials and Methods

3.1 Participants and sample collection

Participants enrolled into the present study were 30 healthy individuals, age between 18 to 20 years old who presented normal white blood cell (WBC) count and absolute neutrophil count. White blood cell count was performed by diluting blood 1:20 with Turk solution, counting under microscope by using counting chamber and calculating WBC in 10^9 cells/L. To determine the WBC differential, a drop of blood was thinly spread over a glass slide, air dried, and stained with the Wright-Giemsa technique. The absolute number of each type of WBC was calculated from the WBC differential and the WBC count. After all participants signed informed consent for use of their samples, 4 ml of intravenous-blood was collected into heparinized BD vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA) at enrolment and transported to the laboratory immediately for further processing.

3.2 Yeast and culture condition

The clinical isolate- *Candida albicans* and industrial strain- *Saccharomyces cerevisiae* were kindly provided by Department of Clinical Microbiology, Faculty of Medical Technology, Rangsit University. All strains were stored as frozen stocks with 25% (v/v) glycerol and routinely cultured on potato dextrose agar (PDA) plates. A pre-culture of each strain was grown on PDA plate and incubated at 30 ^oC for 24–48 hours. A single colony was further sub cultured on PDA plates and incubated at 30 ^oC for 48 hours and used for preparation of cell suspension.

3.3 Preparation of live *Candida albicans* (CL), live *Saccharomyces cerevisiae* (SL) and heat-treated *Saccharomyces cerevisiae* (SH) in suspension

After incubation, each yeast cells on PDA were harvested, washed twice with phosphate buffer (pH 7.2) by centrifugation. By using the preparation of homogeneous fungal suspensions modified from Guidry, & Trelles (1962), the cell suspension of *Candida albicans* and *Saccharomyces cerevisiae* was adjusted to an optical density (OD600nm) of 5.0 and used as CL and SL, respectively. For SH preparation, 5 mL of *Saccharomyces cerevisiae* cell suspension was heat-treated in hot air oven at 190 ^oC for 20 minutes and further adjusted to be the previous volume.

3.4 Phagocytosis activity study

To study the activity of phagocytosis of neutrophils against CL, SL and SH, the attempts were done by modifying the method described previously (Minarova et al., 2021). Briefly, one mL of heparinized whole blood taken from 30 volunteer individuals and 100 µL of live *Candida albicans* (CL), live *Saccharomyces cerevisiae* (SL) or heat-treated *Saccharomyces cerevisiae* (SH) were mixed together and incubated at 37 ^oC in water bath with

shaking condition. At incubation time of 0, 5, 10, 15, 20, 25 and 30 minutes, respectively, blood samples were taken for preparation of thin blood smear. Then, fixed the blood smears with methanol, flooded and stained with Wright Giemsa dye for 4 minutes. Followed by flooding with phosphate buffer pH 6.8-7.2, let slides covered in the buffer for 4 minutes and rinsed slide with running tap water. Then, differential count of neutrophils with and without phagocytosis as well as the numbers of ingested yeast were determined from a total number of 50 neutrophils. The phagocytosis percentage and phagocytosis index that represented an average numbers of the ingested yeast per one neutrophil were calculated by using the following calculation equations, respectively.

Phagocytosis percentage (%) = numbers of neutrophil with phagocytosis x 100/50

Phagocytosis index = total numbers of ingested yeast/50

3.5 Statistic analysis

Data were analysed using IBM SPSS ((An IBM Company, USA). Descriptive statistics were performed for range, average, standard deviation, median, and interquartile range of phagocytosis percentage and phagocytosis index. Inferential statistics were used for comparison study among phagocytosis percentage and phagocytosis index by using Wilcoxon signed-rank test for matched pair difference.

3.6 Ethics

This study was approved by the Ethics Review Board for Human Research of Rangsit University, Thailand (Certificate of approval number: RSUERB2023-048).

4. Results

4.1 White blood cell count and absolute neutrophil count

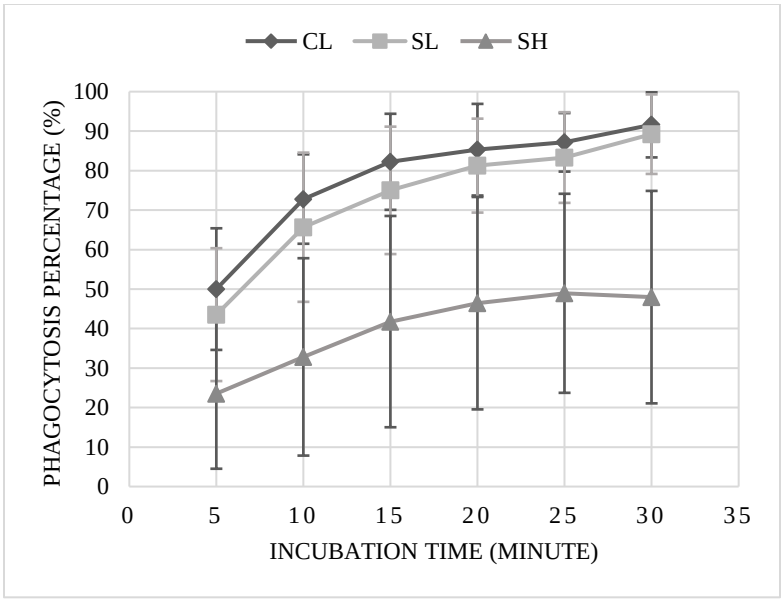
To reduce the effect of low numbers of the white blood cell count and absolute neutrophil count on phagocytosis activity, all blood samples were used in this study should be within normal value. As shown in table 1, all 30 healthy volunteers showed normal white blood cell count and absolute neutrophil count. The range of white blood cell count and absolute neutrophil count were 4.85 – 9.7 x 10^9 cells/L and 2.76-6.05 x 10^9 cells/L, respectively.

4.2 Phagocytosis activity of the neutrophils against CL, SL and SH

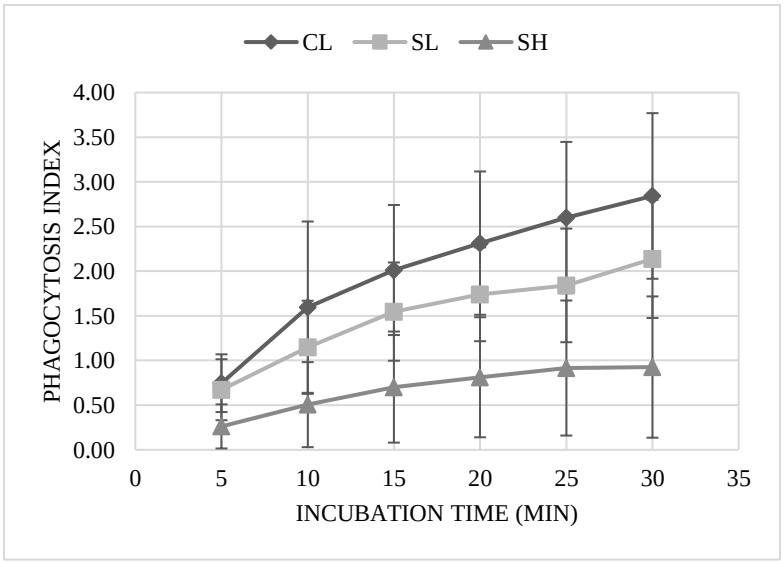
Phagocytosis activities of the neutrophils against CL, SL and SH were illustrated as phagocytosis percentage and phagocytosis index at 0, 5, 10, 15, 20, 25, and 30 minutes of incubation as shown in Figure 1. There was no phagocytosis against CL, SL, and SH at 0 minute. At 30 minutes of incubation, the highest phagocytosis percentage was 91.61%, 89.23%, 57.97% and the highest phagocytosis indexes were 2.84, 2.14 and 0.93 for CL, SL and SH, respectively. Because some neutrophils lysed at 30 minutes of incubation, therefore the optimal reading time seemed to be at 25 minutes of incubation as illustrated in figure 2.

Table 1 White blood cell count and absolute neutrophil count of 30 healthy volunteers

	Range [x10^9 cell/L]	**Mean [x10^9 cell/L]**	**Standard Deviation [x10^9 cell/L]**	**Normal value [x10^9 cell/L]**	**References**
White blood cell count	4.85-9.7	8.51	0.88	4.5 – 11	Cheng et al., 2004
Absolute neutrophil count	2.76-6.05	5.03	0.66	2.0 – 8.0	Pagana et al., 2019
Absolute lymphocyte count	1.44-3.74	2.82	0.59	1.0 – 4.0	
Absolute eosinophil count	0-0.37	0.21	0.10	0.05-0.5	
Absolute basophil count	0-0.017	0.015	0.04	0.025-0.1	
Absolute monocyte count	0-0.14	0.43	0.33	0.1 - 0.7	

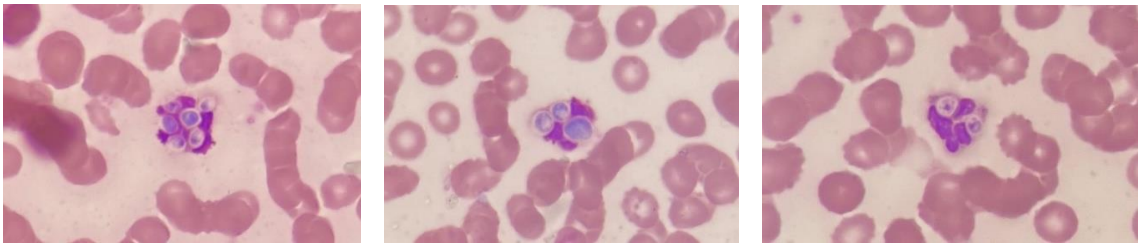


(a)



(b)

Figure 1 Mean and standard deviation of phagocytosis percentage (a) and phagocytosis index (b) of neutrophils against live *Candida albicans* (CL), live *Saccharomyces cerevisiae* (SL) and heat-treated *Saccharomyces cerevisiae* (SH) at 0, 5, 10, 15, 20, 25, and 30 minutes of incubation (n = 30)



(a) (b) (c)

Figure 2 The examples of neutrophils showing phagocytosis against CL (a), SL (b) and SH (c) at 25 minutes of incubation

Table 2 Comparison of phagocytosis percentage of neutrophils against CL, SL and SH at various times of incubation

Incubation time [minutes]	Median [inter-quartile range] of percentage			p-value		
	CL	SL	SH	a	b	c
5	48.0 [39.5-56.0]	44.0 [31.0-56.5]	16.0 [5.5-44.0]	0.089	0.000*	0.000*
10	74.0 [63.5-80.0]	69.0 [52.5-80.5]	19.0 [14.0-50.0]	0.025*	0.000*	0.000*
15	86.0 [73.5-92.0]	77.0 [67.0-88.0]	32.0 [22.5-72.0]	0.011*	0.000*	0.000*
20	88.0 [77.5-94.5]	82.0 [72.0-90.5]	37.0 [22.0-74.5]	0.022*	0.000*	0.000*
25	90.0[85.0-97.0]	84.0 [78.0-91.0]	43.0 [28.0-74.5]	0.020*	0.000*	0.000*
30	94.0[85.0-98.0]	92.0 [86.8-94.5]	42.0 [30.5-77.0]	0.087	0.000*	0.000*

[a] p-value: CL compare to SL, [b] p-value: CL compare to SH, [c] p-value: SL compare to SH

Table 3 Comparison of phagocytosis index of neutrophils against CL, SL and SH at various times of incubation

Incubation time [minute]	Median [inter-quartile range] of index			p-value		
	CL	SL	SH	a	b	c
5	0.66[0.52-1.00]	0.63 [0.39-0.95]	0.16 [0.06-0.51]	0.156	0.000*	0.000*
10	1.30 [1.12-1.77]	1.06 [0.68-1.57]	0.27 [0.16-0.89]	0.002*	0.000*	0.000*
15	1.94 [1.59-2.35]	1.50 [1.18-1.87]	0.42 [0.25-1.35]	0.000*	0.000*	0.000*
20	2.29 [1.62-2.92]	1.60 [1.39-2.06]	0.50 [0.26-1.50]	0.000*	0.000*	0.000*
25	2.62 [1.83-3.26]	1.90 [1.46-2.14]	0.57 [0.40-1.48]	0.000*	0.000*	0.000*
30	2.65 [2.19-3.58]	2.05 [1.63-2.70]	0.59 [0.34-1.47]	0.000*	0.000*	0.000*

[a] p-value: CL compare to SL, [b] p-value: CL compare to SH, [c] p-value: SL compare to SH

4.3 Comparison of phagocytosis activity of the neutrophils against CL, SL and SH

At 25 minutes of incubation, the median [interquartile range] of phagocytosis percentage against CL, SL and SH were 90.0 [85.0-97.0] %, 84.0 [78.0-91.0] % and 43.0 [28.0-74.5] % and median [interquartile range] of phagocytosis index against CL, SL and SH were 2.62 [1.83-3.26], 1.90 [1.46-2.14] and 0.57 [0.40-1.48], respectively. It was found that the median of phagocytosis percentage against CL was higher SL at 10, 15, 20, 25 minutes of incubation and the median of phagocytosis index against CL was higher SL at 10, 15, 20, 25, 30 minutes of incubation (p-value < 0.05). The median of phagocytosis percentage and phagocytosis index of both CL and SL were significantly higher than SH (p < 0.05), as shown in table 2 and 3, respectively.

5. Discussion

To control the variability of phagocytosis, 30 volunteers whose white blood cell count and absolute neutrophil count were within normal limit were included (table 1). Unless specified otherwise, the condition for phagocytosis study in this study was mixing 100 µL of yeast cell suspension adjusted optical density at 600 nm to be 5 with 1 mL

heparinized whole blood, incubated at 37 ºC in water bath with shaking condition. By using this condition, the phagocytosis percentage against all yeast saturated at 25 -30 minutes (figure 1). Phagocytosis activity of neutrophils against live *Candida albican* in this study seems to be faster rate than previously report. Schuit (1979) reported the highest phagocytosis percentage after mixing 1:1 ratio of 1x 10^6 cells phagocytic cells and 1x 10^6 cells yeast suspension for 90 - 120 minutes of incubation. This faster rate of phagocytosis observed in this study may result from some opsonins such as complement in heparinized whole blood enhance phagocytosis process as reported previously (Solomkin et al., 1978). Live *Candida albicans* could be phagocytosed by neutrophils more than live *Saccharomyces cerevisiae* in terms of both percentage and index. These might result from pattern recognition receptors (PRRs) of neutrophils could recognize several corresponding pathogen-associated molecular patterns (PAMPs) on Candida cell wall, such as β-glucan, α-mannan, phospholipomannan and β-mannosides. These PAMPS components are mainly recognized extracellularly by Toll-like receptors and C-type lectin receptors on the host cell surface and lead to different downstream signaling, such as chemokine/cytokine production and phagocytosis. Moreover, once Candida is internalized or phagocytosed, the fungal PAMPs can further activate TLR9 activation intracellularly (Cheng et al., 2012). Whereas *Saccharomyces cerevisiae* consists of two main PAMPS, β- glucan and α-mannan. Although median of phagocytosis percentage and phagocytosis index of both CL and SL were significantly higher than SH, SH was still a phagocytosis trigger. This low phagocytosis activity against heat-treated *Saccharomyces cerevisiae* might result from an effect of high temperature either on reduction of mannan which is a polysaccharide binding to heat labile protein components (Javmen et al., 2017) or the differences in α-mannan and β-1,3-glucans chain lengths that affected their capacity to be recognized by PRRs (Rawling et al., 2023).

6. Conclusions

Although the phagocytosis activities of neutrophils against live *Candida albicans* and *Saccharomyces cerevisiae* are higher than against *Saccharomyces cerevisiae* (heat-treated at baking temperature), heat-treated *Saccharomyces cerevisiae* still has capability for stimulating neutrophil phagocytosis activity. The results from this research are interesting and should be studied further, for example in animal models with chronic inflammation.

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