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3 **Enhanced Immune Response by Vacuoles isolated from**
4 ***Saccharomyces cerevisiae* in RAW 264.7 Macrophages**

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1 **ABSTRACT**

2 Vacuoles are membrane vesicles in eukaryotic cells, the digestive system of cells that break
3 down substances absorbed outside the cell and digest the useless components of the cell itself.
4 Researches on anti-cancer and intractable diseases using vacuoles are being actively conducted.
5 The practical application of this study to animals requires the determination of the biocompatibility
6 of vacuole. In the present study, we evaluated the effects of vacuoles isolated from *S. cerevisiae* in
7 RAW264.7 cells. This showed a significant increase in the production of nitric oxide produced by
8 macrophage activity. Using Reactive Oxygen Species (ROS) Assay, we identified that ROS is
9 increased in a manner dependent on vacuole concentration. Western blot analysis showed that
10 vacuole concentration-dependently increased protein levels of inducible nitric oxide synthase
11 (iNOS), cyclooxygenase-2 (COX-2). Therefore, iNOS expression was stimulated to induce Nitric
12 oxide (NO) production. In addition, pro-inflammatory cytokines levels promoted, such as
13 interleukin 6 and tumor necrosis factor - α . In summary, vacuoles activate the immune response of
14 macrophages by promoting the production of immune-mediated transporters NO, ROS, and pro-
15 inflammatory cytokines.

16 Keywords: *Saccharomyces cerevisiae*, vacuole, RAW 264.7, Cytokine, immunostimulatory
17 activity

1. INTRODUCTION

The immune system is a body defense system designed to protect the body from various externally dangerous invaders, such as viruses, bacteria, and microorganisms, such as fungi, bacteria, and parasites. (1) Immune cells can be divided into innate immunity, which is a non-specific natural immunity, and adaptive immunity, which is responsible for an immune-specific memory response that is formed by memory in response to pathogens. Innate immune cells first attack foreign substances that have invaded the body, and transfer the information obtained from macrophages to the acquired immune cells. Secondly, the T cells and B cells that received the information attack the second with information about foreign substances that re-infiltrated.

The activation of macrophages plays an important role in regulating inflammation and tissue repair.

(2) Macrophages are important components of the immune system, and in addition to acting as host defenses, activated macrophages produce oxidative mediators of reactive oxygen species (ROS) and nitric oxide (NO), and pro-inflammatory cytokines interleukin (IL) or tumor necrosis factor (TNF). (3) Interleukin is an immunomodulatory cytokine group that is first expressed by leukocytes, of which IL-6 is secreted from various cells, such as macrophages, lymphocytes, and keratinocytes, and is involved in acute inflammatory responses. TNF is a cytokine that is involved in the mechanism of biodefense through inflammation and has anti-tumor activity and regulation of differentiation proliferation. (4)

The Vacuole is the most dynamic organelle in eukaryotic cells and is essential for numerous physiological functions. Vacuoles extracted from Yeast *Saccharomyces cerevisiae* are counterparts of mammalian lysosomes. Inside, they have a hydrolase activity optimal at low pH, that plays an

1 important role in the digestion of extraneous foreign substances in cells. (5)

2 Various studies have been conducted for use as a therapeutic agent, such as an eco-friendly
3 antibacterial agent using vacuole, or an anticancer agent using lysosome. (6, 7) To apply this
4 treatment to animals or humans, we tried to determine the effect of vacuoles on the immune system.
5 This study conducted to measure the immune response of macrophages treated with yeast vacuoles,
6 an environment-friendly substance, against RAW 264.7 macrophages. The effect of activating the
7 immune response of RAW 264.7 cells treated with yeast vacuoles was investigated measuring
8 inflammatory mediators such as NO, ROS and iNOS, COX 2 protein, and the pro-inflammatory
9 cytokines TNF α and IL 6.

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2. MATERIALS AND METHODS

2.1 Materials

Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Life Technologies Corp., USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharide (LPS, *Escherichia coli* O111:B4), and Dexamethasone (DEX) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits of TNF- α , and IL-6 were purchased from R&D Systems (Minneapolis, MN, USA)

2.2 Isolation of vacuole from *Saccharomyces cerevisiae*.

Yeast *Saccharomyces cerevisiae* was cultured in YPD medium (1% yeast extract, 2% peptone and 2% D-glucose) and at an OD_{600} of 0.8–0.9, and the culture was obtained by centrifugation at 3,500 rpm for 5 minutes. And recombinant yeast was grown in synthetic dropout (SD) medium at 30 °C incubator. After 24 h of grown in SD medium (0.67% yeast nitrogen base, 0.5% casamino acid, and 2% D-glucose), 2% D-galactose was added and incubated at 180 rpm for 20 h. After adding 0.1 M Tris-SO₄ buffer (Tris-SO₄ (pH 9.4), 10 mM dithiothreitol (DTT)) to the obtained cells, the cell walls of the yeast were incapacitated by incubation at 90 rpm for 15 min in a 30 °C incubator. The reaction was completed, the cells were centrifuged at 3,000 rpm for 5 min, and the supernatant was discarded. The glass beads were added in the equal to the cell amount, and re-suspended in a breaking buffer (20 mM Tris-HCl (pH 7.4), 0.6 M sorbitol, 1 mM phenyl methane sulfonyl fluoride

1 (PMSF)), followed by vortexing 10 times 1 min (on/off) for a total of 20 min. After centrifugation
2 at $500\times g$ for 10 min, the supernatant was subdivided into a microtube, and then centrifugation
3 was conducted at $20,000\times g$ for 30 min by microcentrifuge. After that, the experiment was
4 progressed out using the obtained pellet. For quantification of the concentration of yeast-derived
5 vacuoles, the enzyme contained in the yeast was extracted through the lysis process. After mixing
6 the obtained vacuole and Lysis buffer (0.1% NP-40, 0.1mM PMSF, 5mM DTT) in a 1:1 ratio,
7 followed by vortexing 10 times 10 sec (on/off) for a total of 10 min. After that, it was incubated
8 on ice for 30 min. By measuring the protein concentration of the obtained protein through the
9 Bradford assay, the concentration of the vacuole was traced back.

10

11 **2.3 Observation of the morphologies of vacuole by Field Emission Scanning Electron** 12 **Microscopy (FE-SEM)**

13 The vacuole morphology from *Saccharomyces cerevisiae* was analyzed through FE-SEM images.
14 The obtained vacuoles were diluted 1:100 in DW and lyophilized for 4 h based on a total volume
15 of 100 μL to form a powder. The prepared sample was observed through FE-SEM (SUPRA40VP,
16 Carl Zeiss, Germany) installed at the Center of University-wide Research Facilities at Jeonbuk
17 National University.

18

19 **2.4 Cell culture and Cell viability assay**

20 The mouse monocyte macrophages cell line RAW 264.7 cells were purchased from the Korean
21 cell line bank (KCLB) and were cultured in DMEM supplemented with 10 % FBS, 100 U/mL

1 penicillin, and 100 µg/mL streptomycin in a humidified 5 % CO₂ atmosphere at 37 °C. MTT assay
2 was performed to investigate the effect of vacuoles extracted from *Saccharomyces cerevisiae* on
3 the viability of RAW 264.7 cells. (8) RAW 264.7 cells were seeded in a 24-well plate (1 × 10⁵
4 cells/well), and cultured for 24 h in DMEM-included 10 % FBS. After treatment, different
5 concentrations of vacuole and LPS (1 µg/mL) or Dexamethasone (1 µg/mL) were incubated for
6 24 h, and then 5 mg/mL of MTT solution was added for 2 h. Finally, the supernatant was removed,
7 and formazan crystal was dissolved, using dimethyl sulfoxide (DMSO). Absorbance was measured
8 at 540 nm by an ELISA plate reader. The group treated with LPS (1 µg/mL) was set as a positive
9 control, while the group treated with Dexamethasone (1 µg/mL) was set as a negative control.

12 **2.5 Measure of nitrite**

13 The NO level released from the medium was measured using Griess Reagent System (Promega
14 CO., Ltd., USA). RAW 264.7 cells were cultured in 100 mm dishes at 2 × 10⁶ cells/well and with
15 vacuole and LPS (1 µg/mL) or DEX (1 µg/mL), were incubated for 24 h. The mixture was mixed
16 with a solution of sulfanilamide in equal volume as 50 µL of the culture supernatant, and reacted
17 for 10 min. Afterward, 50 µL of the N-1-naphthylethylenediamine dihydrochlorid (NED) solution
18 was dispensed in each well. Absorbance was measured at 540 nm with an ELISA plate reader. The
19 positive group was set as the LPS (1 µg/mL) treatment group.

1 **2.6 Western-blots analysis**

2 RAW 264.7 cells (2×10^6 cells/well) were seeded in 100 mm dishes and incubated for 24 h. After
3 starvation for 12 h in serum-free DMEM, they were treated with each concentration of vacuole
4 and LPS (1 $\mu\text{g}/\text{mL}$), and Dexamethasone (1 $\mu\text{g}/\text{mL}$) for 24 h. After removing the medium, cells
5 were washed twice with cold DPBS. They were then lysed with cell lysis buffer, and 20 μg of
6 protein concentration was quantified by Bradford assay. Proteins were separated in SDS-PAGE
7 and transferred from Gel to nitrocellulose membranes by electroblotting. Then membranes were
8 blocked in 5 % skim milk in T-TBS for 1 h. The iNOS (Abcam, 1:3000) and COX-2 (Abcam,
9 1:5000) Primary antibody were diluted in 5 % skim milk and incubated overnight at 4 °C. The
10 membranes were washed with TBS-T and reacted with secondary antibody (Abcam, 1:5000) in 5%
11 skim milk at room temperature (RT) for 1 h. The target protein was confirmed by enhanced
12 chemiluminescence (Dyne bio, Korea). The positive group was set as the LPS (1 $\mu\text{g}/\text{mL}$) treatment
13 group.

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15 **2.7 Secretion of TNF- α , IL-6**

16 After treatment for each concentration of vacuole, levels of TNF- α , and IL-6 were measured using
17 an enzyme-linked immunosorbent assay (ELISA) kit (R&D system, Minneapolis, MN, USA).
18 After adding 50 μL of ELISA diluent to each well of the antibody-coated 96-well plate, 50 μL of
19 sample and diluted standards were added, and cells were incubated for 2 h at RT, then washed 5
20 times with washing buffer. Enzyme working reagent was added to each well of 100 μL and
21 incubated for 30 min at RT. After washing a total of 7 times, 100 μL of TMB One-Step Substrate
22 Reagent was added, and the plate was incubated for 30 min at RT. After adding 50 μL of stop

1 solution, the absorbance was measured at 450 nm with an ELISA plate reader. The positive group
2 was set as the LPS (1 $\mu\text{g}/\text{mL}$) treatment group.

3 4 **2.8 Measure of ROS generation**

5 The degree of Reactive Oxidative Species (ROS) occurrence was determined using the 2'-
6 7-dichlorofluorescein diacetate (DCFH-DA) staining method. RAW 264.7 cells were cultured in 6-
7 well plates (1.5×10^5 cells/well) for 24 h. After treatment of vacuole by concentration, the cells
8 were incubated for 24 h. After treatment with 10 μM DCFH-DA, cells were protected from light,
9 and incubated for 1 h. After the obtained cells were scraped, the Lysis buffer (20 mM Tris-Cl (pH
10 7.4), 1 mM EDTA (pH 8.0), 150 mM NaCl, 1 mM EGTA (pH 8.5), 1 % (v/v) Triton X-100) was
11 dispensed in 60 μL increments, followed by a vortexing process of 10 sec (on/off) for a total of 10
12 min. Then DCFH-DA Fluorescence was measured using GloMax[®] Explorer Multimode
13 Microplate Reader (Emission Filter 500–550, Excitation Filter: Blue 475 nm).

14 15 **2.9 ROS-generated fluorescence imaged using confocal laser scanning microscopy**

16 RAW 264.7 cells were cultured in 6-well plates (1.5×10^5 cells/well) on a cover glass for 24 h.
17 After treatment of vacuole by concentration, the cells were incubated for 24 h. Then, 10 μM
18 DCFH-DA was protected from light and incubated for 2 h. After washing once with DPBS, it was
19 treated with Lyso-tracker 100 nm concentration for 20 min. Samples were fixed in 4%
20 paraformaldehyde for 10 min at room temperature and then analyzed by confocal laser scanning
21 microscopy (LSM 880 with Airyscan, Carl Zeiss, Germany).

22

1 **2.10 Data analysis**

2 Each data point was obtained from three independent samples conducted simultaneously for error
3 analysis. The averages are reported with the standard deviations and correlations for several
4 experimental conditions. The data were analyzed using a one-way ANOVA and Tukey test using
5 SigmaPlot (Systat Software, Inc., USA). A p-value < 0.05 was considered significant.
6

1 **3. RESULTS**

2 **3.1 Effect of vacuole on cell viability**

3 The vacuoles extracted from yeast *Saccharomyces cerevisiae* was observed by Field Emission
4 Scanning Electron Microscopy (FE-SEM) (Fig. 1A). The vacuoles identified by FE-SEM were
5 observed to have a round shape and a size of 200 nm. MTT assay was performed to evaluate the
6 cytotoxicity of vacuole extracted from *Saccharomyces cerevisiae* to RAW 264.7 macrophages (Fig.
7 1B). Vacuoles were treated at various concentrations of (5, 10, 20, 40, 120, 200, and 400) $\mu\text{g/mL}$
8 in RAW 264.7 cells, and incubated for 24 h. Fig. 1 B shows that the vacuoles revealed cell viability
9 of $(81.42 \pm 5.3) \%$ at 20 $\mu\text{g/mL}$ concentration, and a cell viability of $(76.88 \pm 2.17) \%$ at 40 $\mu\text{g/mL}$
10 concentration compared to the control group. Therefore, vacuole with cell viability of 80 % or
11 higher were selected for subsequent experiments at a concentration of 20 $\mu\text{g/mL}$ or less.

13 **3.2. Effect of vacuole on the Nitric oxide (NO) production and protein expression of iNOS, 14 COX-2**

15 Nitric oxide is a cell-signaling molecule that works in many biological processes. (9) Nitric oxide,
16 produced by macrophages, is a signal transduction molecule that acts as a defense against infected
17 microorganisms in cells and has been demonstrated to inhibit the proliferation inhibitory activity
18 of bacteria and cancer cells, and nonspecific sedative defense mechanisms. Griess assay was
19 performed to investigate the effect of vacuole on NO production in RAW264.7 cells (Fig. 2A).
20 Reaction for 24 h after treatment with each concentration (5,10 and 20 $\mu\text{g/mL}$) of vacuole and LPS
21 (1 $\mu\text{g/mL}$) and Dexamethasone (1 $\mu\text{g/mL}$) confirmed that the production of NO increased,

1 depending on the concentration of vacuoles. In addition, nitric oxide synthase (NOS) is an enzyme
2 that promotes the production of NO from L-arginine, and can be broadly classified into constitutive
3 NOS (cNOS), and inducible NOS (iNOS). Of these, iNOS is synthesized by inflammatory stimuli,
4 and produces large amounts of NO. (10) Then, a western blot was performed to confirm the effect
5 of vacuole on RAW264.7 macrophages and iNOS and COX-2 protein expression (Fig. 2B). The
6 iNOS and COX-2 protein expression levels increased with the concentration of vacuoles, which
7 showed a correlation with NO and iNOS protein expression levels. Furthermore, in order to find
8 out which components of vacuoles extracted from wild-type yeast activate RAW 264.7 cells,
9 enzymes and pellets extracted from vacuoles were treated under equal conditions (Fig. 2D). In the
10 group treated with enzyme, the expression of iNOS protein was not observed; but in the group
11 treated with pellet, iNOS protein was expressed. We investigated whether vacuoles penetrate into
12 RAW 264.7 cells by treatment with the vacuole extract of recombinant yeast strain YPT7 (Fig.
13 2C). YPT7 protein is located in the membrane of the vacuole and a transformed yeast recombinant
14 plasmid with GFP attached, pYES2.0 :: YPT7 :: GFP was used. (7) At this time, the location of
15 the vacuole can be confirmed through the signal of GFP. When the vacuoles extracted from
16 recombinant yeast were treated in RAW264.7 cells for 24 hours, it was confirmed that the vacuoles
17 did not penetrate into the cells. Therefore, the vacuoles were activated by the membrane
18 component of the vacuoles, without penetrating into RAW 264.7 cells.

20 **3.3. Effect of vacuole on the levels of TNF- α , IL-6**

21 Cytokines are carriers of the immune system, and are regulatory proteins that can be produced in
22 eukaryotic cell types. They are also a factor that modulates the immune response by acting on the

1 cells and hematopoietic cells involved in the host defense and damage healing process. (11) TNF-
2 α is a cytokine that is mainly produced in activated macrophages, and is responsible for anti-tumor
3 activity and anti-microbial activity regulation of differentiation and proliferation. (4) RAW 264.7
4 cells were treated for 24 h with either vacuoles (5,10 and 20 $\mu\text{g}/\text{mL}$) or LPS (1 $\mu\text{g}/\text{mL}$) and pro-
5 inflammatory cytokines TNF- α and IL-6 were quantified using a mouse ELISA kit. (Fig. 3). As
6 a result, vacuoles significantly increased the production of the pro-inflammatory cytokine TNF- α
7 (Fig. 3A). It was confirmed that the production of TNF- α by vacuole increased 6-fold at a
8 concentration of 10 $\mu\text{g}/\text{mL}$ compared to the control group (0 $\mu\text{g}/\text{mL}$ vacuole treatment). IL-6
9 affects adaptive immunity, and is a cytokine related to the proliferation of B cells and the secretion
10 of antibodies, and functions in the immune response and acute inflammatory response. (12) The
11 production of IL-6 by vacuole increased in a concentration-dependent manner (Fig. 3B). (13)

12

13 **3.4. Effect of vacuole on ROS generation in RAW 264.7 cells**

14 Reactive oxygen species (ROS) are produced in processes that are activated in normal cells and
15 are involved in biological processes, including cell differentiation and the degree of response to
16 cytokines. (14) The ROS by vacuole in RAW 264.7 cells was detected using 2'-
17 7'dichlorofluorescein diacetate (DCFH-DA) staining (Fig. 4). LPS (1 $\mu\text{g}/\text{mL}$) was set as a positive
18 control. The vacuoles treatment group significantly induced the generation of ROS (Fig. 4A). As
19 a result of Confocal laser scanning microscopy imaging of the expression level of ROS, vacuole
20 concentration was the highest at 10 $\mu\text{g}/\text{mL}$ (Fig. 4B).

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22

1 DISCUSSION

2 By using the *Saccharomyces cerevisiae*-derived vacuole used in this study, it was possible to target
3 cancer and effectively deliver anticancer drugs, and research is being actively conducted as new
4 therapeutic material such as increasing anticancer and antibacterial effects through gene
5 recombination. (7, 15) However, studies on the immunological effects of *Saccharomyces*
6 *cerevisiae*-derived vacuoles are insufficient. In this study, the effect of *Saccharomyces cerevisiae*-
7 derived vacuoles on the immune activation effect of mouse macrophages was investigated.
8 Macrophages are produced from monocytes and are the main cells responsible for innate immunity.
9 (16) Macrophages play several important roles in the immune system. First, free radicals are
10 produced and secreted to kill bacteria, and external bacterial proteins degraded in macrophages are
11 presented on their surface, and information of foreign substances invading T cells is transmitted.
12 (17) Among the immune response mediators produced at this time, various substances such as
13 nitric oxide (NO), reactive oxygen species (ROS), histamine, and cytokines are included. The NO
14 produced at this time plays an important role in protecting the body as a cell-signaling molecule.
15 The most important function is to relax the internal muscles of blood vessels by dilating blood
16 vessels, regulate blood pressure, and perform various physiological functions such as inhibition of
17 platelet aggregation, immune regulation, and induction of apoptosis. Although excessively
18 produced NO shows cytotoxicity to normal cells, an appropriate amount of NO has resistance to
19 foreign antigens and plays an important role in host protection. (16) In addition, inducible nitric

1 oxide synthase (iNOS) is produced in large amounts for host protection when stimulated from the
2 outside, and as a result, the production of NO is induced. In this study, it was confirmed that when
3 RAW 264.7 cells were treated with vacuoles extracted from *Saccharomyces cerevisiae*, NO
4 production was promoted and iNOS protein expression also increased in a vacuole concentration-
5 dependent manner. (Fig. 2 A, B) Moreover, among pro-inflammatory cytokines, TNF- α is a
6 cytokine that plays an important role in several immune-mediated inflammatory diseases. TNF- α
7 is mainly produced in activated macrophages and functions to enhance antitumor activity and the
8 ability to kill infected cells. (17) IL-6 is a cytokine that plays an important role in acute-phase
9 response, inflammation, hematopoiesis, and progression of cancer, and performs the body's
10 defense function against antigens introduced from the outside. (18) In this study, the effect of
11 vacuole on the production of pro-inflammatory cytokines TNF- α and IL-6 was studied. As a result,
12 it was shown that vacuole induces TNF- α and IL-6 production. These results suggest that vacuoles
13 activate macrophages and upregulate immune capacity. While Reactive oxygen species (ROS)
14 plays an important role in protecting the living body by sterilizing foreign substances invading the
15 body, excessively generated ROS can cause aging and attack normal cells. The vacuoles treatment
16 group significantly induced the generation of ROS. As a result of confirming the generation of
17 ROS by vacuoles through confocal fluorescence images, compared with the LPS-treated group,
18 the expression of ROS was the highest at the concentration of 10ug/ml of the vacuoles. In this
19 study, it was confirmed that this immune activation effect is caused by the membrane component
20 of the vacuole, but it cannot be determined exactly which component is induced in this study.
21 Activation of macrophages by vacuole membrane components requires additional component
22 analysis studies. Lipopolysaccharide (LPS) used as a positive control used in this study is well

1 used as a strong immune response inducer, but it is difficult to practically apply to the human body
2 as an immune enhancer due to its strong toxicity. Compared to LPS, vacuoles have significantly
3 lower toxicity and are judged to have excellent immune-enhancing effects, suggesting potential as
4 a functional material for strengthening immunity.

6 **4. CONCLUSION**

7 In this study, after treating vacuoles extracted from *Saccharomyces cerevisiae* with RAW 264.7
8 macrophages, cell viability, NO and ROS production, and pro-inflammatory cytokines, such as
9 IL-6 and TNF- α were measured, and the following results were obtained. The cell viability at
10 vacuole concentrations of (5,10, and 20) $\mu\text{g/mL}$ was greater than 80 %. It was confirmed that in
11 the production of NO, the vacuole increased at all concentrations. Vacuole was also correlated
12 with the results of NO in iNOS and COX-2 protein expression. The significant increase of
13 production of pro-inflammatory cytokines IL-6 and TNF- α and ROS at all concentrations of
14 vacuoles was confirmed. Therefore, the vacuole extracted from *Saccharomyces cerevisiae*
15 suggests that the immune response is activated by promoting the increase of the immune mediator
16 in RAW 264.7 cells.

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3 **Data Availability Statements**

4 All data generated or analyzed during the present study are included in this article.

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1 **FIGURE LEGENDS**

2 **Figure 1.**

3 **Effect of vacuoles on the cell viability of RAW 264.7 cells.** (A) Field Emission Scanning Electron Microscopy
4 (FE-SEM) imagery of vacuole extracted from *Saccharomyces cerevisiae*. (B) Treatment with various
5 concentrations of vacuole for 24 h. LPS (1 µg/mL) was used as a positive control. (Values are the mean ± SD of
6 three independent experiments).

7 **Figure 2.**

8 **Effect of vacuoles on the production of inflammatory mediator.** Effect of vacuole on (A) NO Production, (B)
9 iNOS and COX-2 Protein Level of RAW 264.7 cells. (C) Treatment with GFP-vacuole; confocal scan confirmed
10 that it did not enter RAW 264.7 cells. (D) Effect of vacuole enzymes and pellets on iNOS protein Level of RAW
11 264.7 cells. * represent p value<0.05, ** represent p value<0.002 compare with the control group.

12 LPS (1 µg/mL) was used as a positive control. (Values are the mean ± SD of the three independent experiments).

13 **Figure 3.**

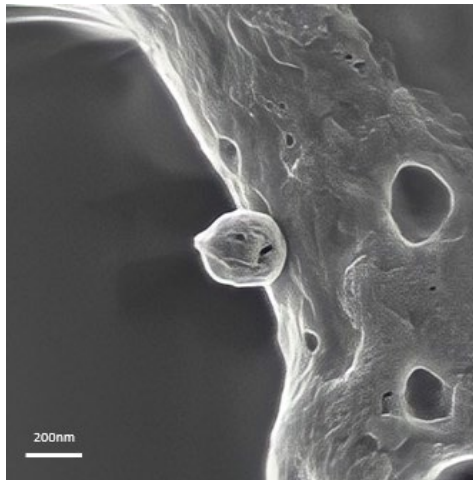
14 **Effect of vacuoles on the production of Pro-inflammatory cytokine.** (A) TNF- α, and (B) IL-6 secretion by
15 vacuole of RAW 264.7 cells. * represent p value<0.05, *** represent p value<0.005 compare with the control
16 group. LPS (1 µg/mL) was used as a positive control. (Values are the mean ± SD of three independent
17 experiments).

18 **Figure 4.**

19 **ROS production by vacuoles in RAW 264.7 cells.** (A) RAW 264.7 cells were exposed at vacuoles for 24h and
20 ROS was detected with DCFH-DA (B) Confocal laser scanning microscopy imaging of the expression level of
21 ROS. LPS (1 µg/mL) was used as a positive control. (Values art the mean ± SD of three independent experiments).

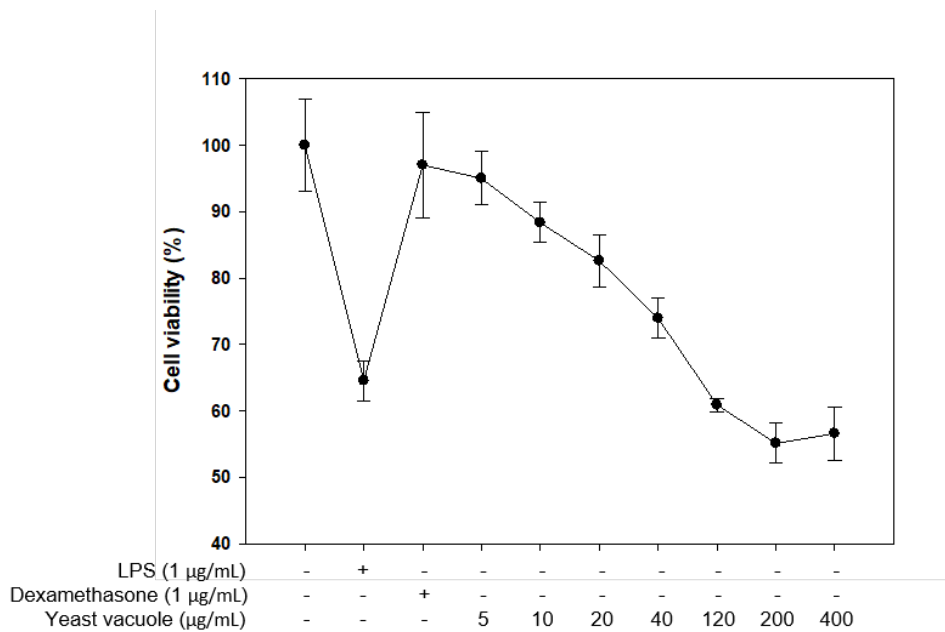
1

2 (A)



3

4 (B)



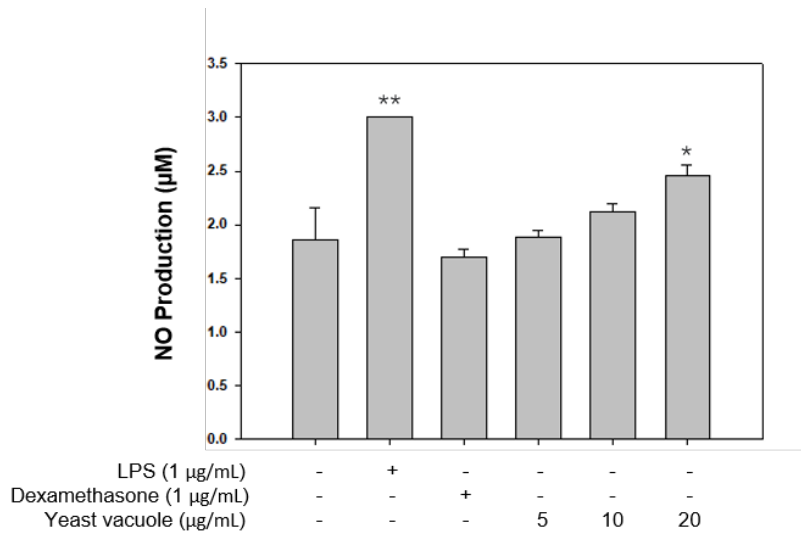
5

6

Figure 1

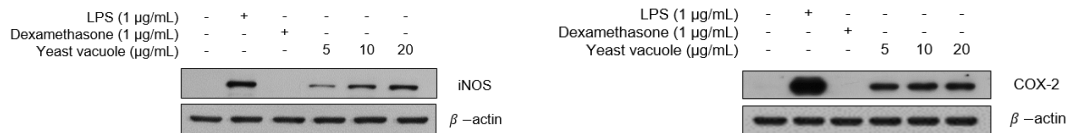
1

2 (A)



3

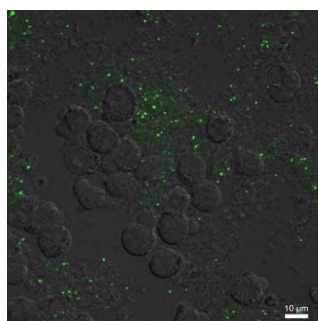
4 (B)



5

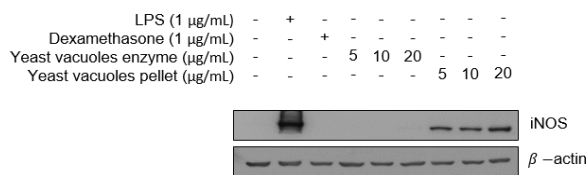
6

7 (C)



8

(D)

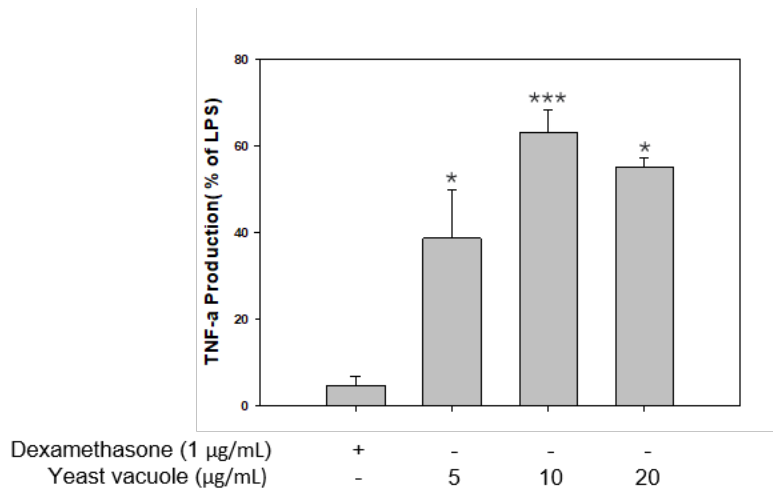


9

Figure 2

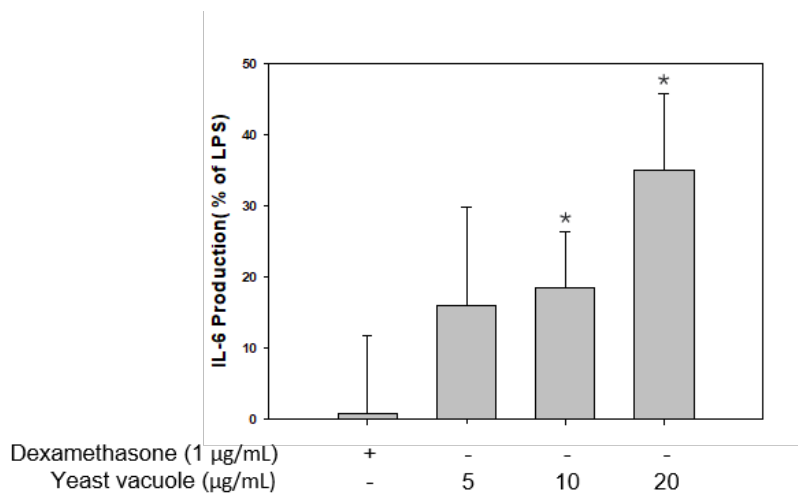
1

2 (A)



3

4 (B)



5

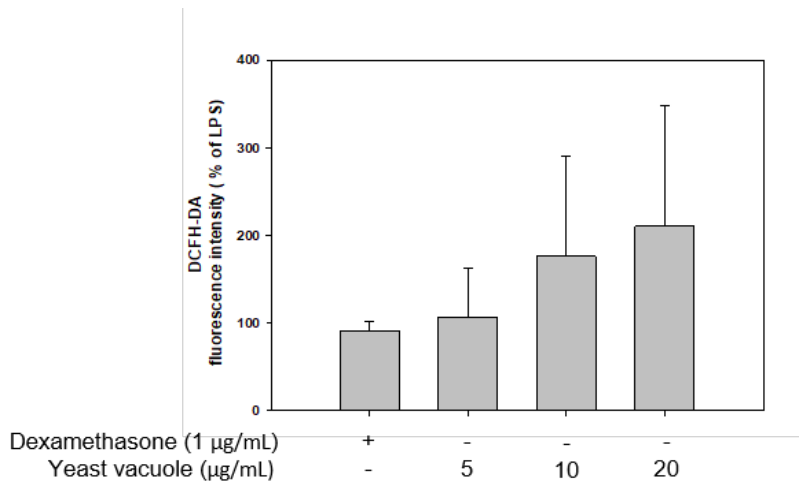
6

7

Figure 3

1

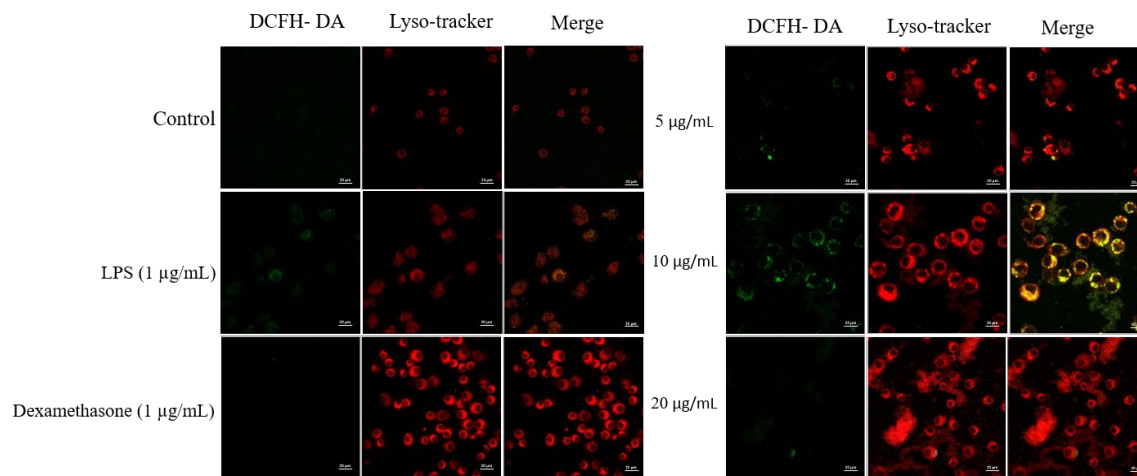
2 (A)



3

4 (B)

5



6

7

8

Figure 4