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Research Article

Immunological effects of *Penicillium digitatum* in rabbits

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Abstract

This study aimed to investigate the immune responses of *Penicillium digitatum* antigens in rabbits. Twenty healthy rabbits were divided into 2 groups (15 for the test group) and (5 for the control group). The test animals were immunized with 500µg/ml of *P. digitatum* spores. The stimulated immune response was determined using Delayed type hypersensitivity-skin, Phagocyte activity (NBT), E-rosette, and leucocyte migration inhibitory factors (LIF) tests. The results of the delayed-type hypersensitivity test showed erythema in the skin thickness in immunized rabbits after 24, 48, and 72 hrs after injection of antigens, and necrosis after 72 hrs. The results showed increases in the percentage of phagocytic activity (74.333±1.329) compared to the control group (40.222±2.709), and a higher number of E-rosette (70.31±1.279) compared with control one (27.6±1.22). The results showed high significant LIF (55.66±2.11) in the treated group as well.

Keywords: Delayed type hypersensitivity-skin test, NBT, E-rosette test, LIF.

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Introduction

Fungal infections are a global threat to human health, causing 1.5-2 million deaths per year (Bongomin et al. 2018) and they are great threat to food safety as well. The deficiency of available antifungals and the emergence of pathogenic fungi resistance to antifungal drugs are crucial concerns in medicine and agriculture (Bugeda et al. 2020). *Penicillium digitatum* is the most significant indoor and outdoor fungus recognized to cause respiration infections. When cell-mediated immunity (CMI), which includes innate and adaptive immune systems cells, is disrupted in immunocompromised patients due to widespread antibiotics or medications. Those spores can germinate and increase in the host, causing toxicity and a variety of clinical outcomes such as pulmonary allergic diseases (AL-bermani et al. 2013).

Penicillium digitatum can be found in soils worldwide, and humans are exposed daily to the spores of this airborne pathogen. Five out of 770 patients with pulmonary lung sickness have been proven to have low antigen-specific precipitating antibodies to *P. digitatum* antigen with Ouchterlony double immuno-diffusion evaluation. In analyses of antigen isolate from patient sputum culture fluid or sputum, neither of those patients had antigen-precise precipitating antibodies to *P. digitatum* (Oshikata et al. 2013). Therefore, this study aimed to investigate the immune responses of rabbits to *P. digitatum.*

Materials and methods

Laboratory animals: Twenty healthy albino rabbits (1.5-1.75kg) were obtained and divided into two groups viz. 15 animals for the treated groups and five animals for control one, and kept in large plastic cages for one week before beginning the experiment.

Intranasal challenge with *P. digitatum* spores: *Penicillium digitatum* was cultured in PDA for 14 days. Their spores were harvested by washing plates with sterile 0.1% tween, resulting in fungal suspension that filtered through two layers of sterile gauze to remove their hyphae (Noverr et al. 2005). The conidia were washed twice with phosphate buffer saline (PBS) and centrifuged at 3500r/m for 5min. The supernatant was removed, and the pellet (conidia) was counted using a W.H.O Opacimeter to 10^8 conidia/ml in PBS before diluting to 10^7 conidia/ml.

The treated rabbits were given 0.5ml of *P. digitatum* conidial suspension weekly via their nostril for six weeks. The blood samples were taken via heart puncture using sterile disposable syringes, as 5ml of blood at the end of the experiment.

Delayed type hypersensitivity skin test: For each animal, 50µl of *P. digitatum* spores were injected into their right footpad, and normal saline into their left footpad (except the control group injected with normal saline). After 24 hours, 48 hours, and 72 hours, delayed form hypersensitivity was measured using a digital Vernia and expressed ad millimeters (Hudsen & Hay 183).

Erythrocyte-rosette formation Test: The E-rosette test was done according to Nafarndam et al. (201). Sheep red blood cells (sRBC) were kept in Alsever's solution at 4°C for 1 week and then used. The cell was washed twice and calibrated to a 5% suspension in N.S. before use. The lymphocyte suspension (0.25ml) was mixed with the sRBC cell suspension (0.25ml) and incubated at 37°C for 15min. The mixed cell suspension was centrifuged for 5min at 1000rpm before incubating at 4°C overnight. The pellet's top layer was softly resuspended by shaking after separating the supernatant fluid. A drop of the cell suspension was put on a slide of glass, which was then covered with a coverslip. Both lymphocytes that bound more than three sRBC were considered positive.

Leukocyte Migration Inhibition Factor (LIF): The migration inhibition was measured according to Khandoga et al. (2009), as follows: Agar-A media (2%t agar) was prepared in a sterile plastic plate, and 2cm diameter wells were created into the agar plate. After centrifugation by hematocrit centrifuge for 10min, each well was filled with capillary tubes containing systemic blood and each well was filled 0.1ml of Eagle basal medium, with one well serving as control. 0.1ml of the antigen prepared in this study was added to one well. In a humid condition, incubation was done at 37°C for 24 hours. LIF by oculometer. LIF was measured using the following equation of LIF = (Diameter of migration circle with antigen / Diameter of migration circle without antigen) × 100

Phagocyte activity test: The test was done according to Park et al. (1982). In a microtiter plate well, 0.1ml of rabbit blood was combined with 0.1ml of NBT solution. The mixture was carefully mixed and wrapped to maintain humidity before being incubated at 37°C for 15-25min. After that, using Wright stain, a smear was prepared and was left for 5min before being rinsed with D.W. and allowed to dry. The prepared slide was examined under a microscope and based on the results, phagocyte activity was calculated according to the flowing equation: Phagocyte activity = (Number of phagocytes reducer to pigment / Number of total phagocytes) × 100

Statistical analysis: The data was analyzed using the Statistical for Social Science (SPSS) 26. The mean and standard deviation were used to display the results. The Chi-square test was used to compare the variations between the frequencies, and the T-test was used to compare the mean values between classes. To find differences between more than two classes of continuous variables, the ANOVA and LSD tests were used. A $P<0.05$ was recognized as

Table 1. Delayed Type Hypersensitivity (DTH)-Skin test of rabbits *Penicillium digitatum*.

Antigen	24 hr.		48 hr.		72 hr.	
	Mean ± SD					
	E	N	E	N	E	N
Test animal	2.106±0.349	-	2.706 ±0.328	-	2.392 ±0.379	+
Control animal	-	-	-	-	-	-

E: Erythema, N: Necrosis

statistical significance (Team 2015).

Results

Table 1 shows the mean diameter of erythema (mm) and necrosis of the skin. The highest diameter of erythema was recorded in the group immunized with antigens after 48 hours (2.706±0.328) and necrosis appeared only after 72 hours. The results revealed a rise in the phagocytic activity of rabbits immunized by *P. digitatum* as its Ag was 74.333±1.329, but the control group's mean value was 40.222±2.709. The results also show a significant increase of the E-rosette test (70.133±1.279) compared to the control one (27.6±1.22). Leukocyte migration inhibitory factor was shown a significant difference (55.666±2.11) compared to the control group (83±2.04) (Table 2).

Discussion

The skin test showed an increase in the diameter of erythema after 48 hours, in agreement with the findings of Farag et al. (2010). The delayed form hypersensitivity skin test is a procedure that helps the diagnosis of infectious diseases. The test measures cutaneous (skin) hypersensitivity to antigens and determines if a person has already been exposed (sensitized) to a specific antigen (Al-Samarrae 2011). The memory cells in modulating Th1 to secrete interferon (INF), a highly effective mediator that activates macrophage migration to the location of the skin's reacted area, is occurred in the skin test (Rosenthal & Tan 2007). Although macrophages secrete Interleukin-1 (IL1), which promotes T cell proliferation and differentiation into T helper-1 (Th1) cells, macrophages secrete Interleukin-2 (IL2), a chemotactic factor that attracts macrophages to activated T cells. The foreign protein was first taken up by antigen-presenting cells, which broke it down into many peptides that bound to the binding site of the MHC class II molecule. This immunogenic peptide is recognized by the T-cell antigen receptor, which secrets lymphokine (CD4+) and is recognized by the antigen–MHCII complex, then transformed into cytotoxic cells (CD8+) that recognize the class I MHC molecule. When a sensitized animal's skin is injected with a certain antigen, an inflammatory response takes several hours to develop activity on the injected site (Mahmood et al. 2015).

Our results showed a high increase in the phagocytic activity of rabbits immunized with *P. digitatum*, similar to Wojcicka-Lorenowicz et al. (2018), who found immunizing rabbits with *Trichophyton mentagrophytes* increases phagocytic activity. The activation of macrophages is regulated by the Fc (fragment crystallizable) receptor, a protein located on the surface of many immune cells, including macrophages. The activation of Fc receptors causes macrophage activation (Mahfudh et al. 2020). When phagocyte cells are exposed to various antigens that interact with the cell membrane, reactive oxygen species such as $O2^-$ and H_2O_2 are generated. The NBT reduction tests the presence of free radicals produced by phagocytes. This method can estimate the degree of (stimulation) normal cells or their ability to stimulate later phagocytosis. NBT is a soluble, yellow-colored electron acceptor. Stimulated neutrophils incorporate the dye composite into phagosomes, which fuse with lysosomes, impacting intracellular reduction in the formation of blue insoluble Formosan crystals (Rheem et al. 2016).

E-rosette is a commonly used simplified method

Table 2. Cellular Immune Response in Rabbits that Immunized with *Penicillium digitatum*.

Test	Test animals		Control animals		
	Mean	SD	Mean	SD	*P*-value
Phagocyte activity	74.333	1.329	40.222	2.709	<0.001
E-rosette	70.133	1.279	27.600	1.22	<0.001
LIF	55.666	2.110	83.00	2.04	<0.001

for quantifying T-lymphocytes. It is not the same as the EAC-rosette test, which is used to measure B-lymphocytes quantitatively. The E-rosette test involves the interaction of antibodies (A), complement (C), sheep erythrocytes (E) and multiple ligands on the surface of B-lymphocytes, while the interaction of sheep erythrocytes and specific T-cell receptors happens directly in the case of T-lymphocytes (CD2) (Quercia et al. 2012). Leukocyte migration inhibitory factor was shown to significantly differ from the control group. LIF was originally described as a T-cell product and later found to be generated by pituitary cells (Riedemann et al. 2004).

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