



Cellular stress and Inflammatory Responses in Mice Exposed to *Aspergillus niger*, *Rhizopus oryzae*, and *Rhizopus delemar*

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SUMMARY. Epidemiological studies have shown that a few hundred million people around the world are exposed to biological agents such as fungi resulting in a number of diseases. In this study, mice were intranasal exposed to *Aspergillus niger*, *A. flavus*, *Rhizopus oryzae*, and *Rhizopus delemar* conidia (25) μ L (107 conidia/mL normal saline) every other day for twenty-eight days, while the control group received normal saline. After 28 days, the liver and kidney were removed for enzyme activity studies. Superoxide dismutase (SOD), Catalase (CAT) and Glutathione (GSH) had the highest enzyme activity under control measuring 1.5U/mL, 30.2U/mL, and 0.22 μ M, respectively. *R. delemar* recorded the highest level of enzyme activity for malondialdehyde at 4.3 μ M, *A. niger* and *A. flavus* had similar total antioxidant capacity (TAC) values at 0.37 mM/g. *R. oryzae* and *R. delemar* recorded 0.39 mM/g and 1.9 mM/g respectively. *R. delemar* recorded the highest activity of LDH measuring 120U/L, the lowest LDH value was recorded under control at 30 U/L. *A. niger*, *A. flavus*, and *R. oryzae* recorded slightly different levels of LDH. The highest level of Hsp70 proteins was recorded 0.62 ng/mL under *A. niger* followed by *A. flavus* at 0.57 ng/mL. *R. delemar*, *R. oryzae*, and control recorded 0.39, 0.28, and 0.17 ng/mL, respectively. In the liver function test, *R. delemar* mice treated recorded the highest values of AST and creatinine compared others. *R. delemar* expressed the highest level of immune responses under IL-6, TGF-B1, and to IgM. The immune responses under IL-10 and NF-KB were expressed at minimal. Immune response under INF-Y (ng/L) was expressed with slight differences for the various fungi isolates.

RESUMEN. Los estudios epidemiológicos han demostrado que unos pocos cientos de millones de personas en todo el mundo están expuestas a agentes biológicos como los hongos que provocan una serie de enfermedades. En este estudio, el modelo de ratón se estableció mediante la administración intranasal de conidias de *Aspergillus niger*, *A. flavus*, *Rhizopus oryzae* y *Rhizopus delemar* (25) μ L (107 conidias/mL de solución salina normal) en días alternos durante veintiocho días, mientras que el grupo de control recibió solución salina normal. Después de 28 días, se extirparon el hígado y el riñón para estudios de actividad enzimática. La superóxido dismutasa (SOD), la catalasa (CAT) y el glutatión (GSH) tuvieron la actividad enzimática más alta bajo control, midiendo 1,5 U/mL, 30,2 U/mL y 0,22 μ M, respectivamente. *R. delemar* registró el nivel más alto de actividad enzimática para el malondialdehído a 4,3 μ M, *A. niger* y *A. flavus* tuvieron valores similares de capacidad antioxidante total (TAC) a 0,37 mM/g. *R. oryzae* y *R. delemar* registraron 0,39 mM/g y 1,9 mM/g respectivamente. *R. delemar* registró la mayor actividad de LDH midiendo 120U/L, el valor más bajo de LDH se registró bajo control a 30 U/L. *A. niger*, *A. flavus* y *R. oryzae* registraron niveles ligeramente diferentes de LDH. El nivel más alto de proteínas Hsp70 se registró con 0,62 ng/mL bajo *A. niger* seguido de *A. flavus* con 0,57 ng/mL. *R. delemar*, *R. oryzae* y el control registraron 0,39, 0,28 y 0,17 ng/mL, respectivamente. En la prueba de función hepática, los ratones *R. delemar* tratados registraron los valores más altos de AST y creatinina en comparación con otros. *R. delemar* expresó el nivel más alto de respuestas inmunes bajo IL-6, TGF-B1 e IgM. Las respuestas inmunitarias bajo IL-10 y NF-KB se expresaron al mínimo. La respuesta inmune bajo INF-Y (ng/L) se expresó con ligeras diferencias para los diversos aislados de hongos.

KEY WORDS: disease, fungi, inflammatory responses, lipid peroxidation, oxidative stress.

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INTRODUCTION

The primary means of survival for the class of eukaryotic creatures known as fungi is parasitism or pythogenesis. They are ubiquitous and can be found in most parts of human body and external habitats like air, water, and soil. Because most fungi are opportunistic pathogens, which means that when the human body is healthy, it has a strong defense system and will not cause human illness, they mostly infiltrate the human body when the immune function of the human body is declining and cause human illness. Epidemiological studies have shown that a few hundred million people around the world are exposed to biological agents such as fungi. Fungi are hard to avoid as millions are found in pillows, leaf surfaces, compost, air conditioning, heating systems, damp houses, and in all locations. They thrive in warm and damp environments, both outdoors and indoors. Fungi cause lung disease by different mechanisms such as allergic asthma^{1,2}, infections (*e. g.* aspergillosis), and inflammation (*e.g.* hypersensitivity pneumonitis, and farmer's lung disease)^{3,4}.

Aspergillosis is a fungal infection caused by spores of indoor and outdoor *Aspergillus* mold species. Due to the ubiquitous nature of *A. niger*, its spores are commonly inhaled by humans from their surrounding environment. However, aspergillosis infection customarily occurs in people with compromised immune systems or pre-existing lung conditions like asthma and cystic fibrosis. *A. niger* has been found to cause physical manifestations in humans. It also has mild opportunistic characteristics of causing opportunistic respiratory infections associated with pneumonia in immunocompromised individuals than the other species of *Aspergillus* such as *A. flavus*, *A. fumigatus*, and *A. clavatus*. In rare cases, however, it can cause opportunistic invasive pulmonary aspergillosis in individuals with immune-compromised systems on inhalation of a damaged epithelial lining and respiratory tract system, causing severe lung disease.

AFB₁ is a metabolite of *Aspergillus flavus* and *A. parasiticus*, which is classified under Group I carcinogenic agents by International Agency for Research on Cancer⁵, and is a potent hepatotoxic and hepatic carcinogenic mycotoxin in humans. The mechanisms of AFB₁-induced liver injury are complex, and previous studies have shown that inflammatory response is a critical step in the process. Mehrzad *et al.*,⁶ found that AFB₁ activates TLR2 and TLR4, which trigger the NF- κ B signaling pathway that leads to the synthesis

and secretion of TNF- α , IL-1 β , IL-6 and other pro-inflammatory cytokines in murine pure primary astrocytes. Cytokines are released during the early phase of an immune response to allergen or foreign substance which triggers cellular inflammatory response over the next few hours which later results in recurrent symptoms in the host^{7,8}. Ingestion is the most common route of exposure to AFB₁. Upon consumption of AFB₁-contaminated meal, AFB₁ is metabolized in the liver by microsomal mixed-function oxidase (MFO), a member of the cytochrome P450 (CYP) superfamily to different metabolites. The liver is the primary organ attacked by AFB₁, and AFB₁-induced liver cell cytotoxicity is involved in the interactions of inflammation, oxidative stress, and liver metabolic enzymes. In rats, there is ample evidence demonstrating the toxicities of AFB₁ to the hepatorenal system.

Inflammation has been traditionally defined according to its main features: redness, warmth, pain, and swelling. However, one of the latest definition of inflammation includes its protective role in response to invading pathogens or endogenous signals, resulting in the elimination of the initial cause of injury, clearance, and tissue repair⁹. Furthermore, the activation and resolution of the inflammatory response are strictly regulated to protect the organism from collateral damages. In this regard, specialized cell-surface receptors known as pattern-recognition receptors (PRRs) activate inflammatory cascades upon the recognition of pathogen-associated molecular patterns (PAMPs) or endogenous signals, such as damage-associated molecular patterns (DAMPs)¹⁰. Consequently, activation of PRRs triggers the release of proinflammatory cytokines and chemokines—which are secreted proteins that exert further pro-inflammatory cascades and recruit additional immune cells to the site of infection¹¹.

On balance, inflammation plays a crucial role in the resolution of fungal infections by alerting the host-immune system of external threats and promoting the clearance of the pathogenic fungi^{9,10}. The aqueous extract of *Rhizopus oryzae* U-1 (RU) increases the number of Th1 cells, which defend against *Salmonella* infection and regulate immunity in rats. Interleukin-1 beta (IL-1 β) has been described to play an important role in response to pathogenic fungi^{12,13}. This interleukin is involved in the induction of other pro-inflammatory proteins, hematopoiesis, differentiation of TH17 cells, and development of IL-10¹¹. The tumor necrosis factor α (TNF- α) mediates the

host-apoptosis and inflammation, where it has a dual role as an activator and immunosuppressor of the inflammatory response¹⁴. A recent study¹⁵ showed that exposure of neutrophils to *R. oryzae* hyphae results in up-regulation in Toll-like receptor 2 expression and in a robust proinflammatory gene expression with rapid induction of NF- κ B pathway-related genes. Although pulmonary alveolar macrophages harvested from lungs of immunocompetent mice are able to ingest and inhibit germination of *R. oryzae* sporangiospores, these bronchoalveolar macrophages have limited capacity to kill the organism *in vitro*. In contrast, pulmonary alveolar macrophages of immunosuppressed mice are unable even to prevent germination of the sporangiospores *in vitro* or after intranasal infection. Endocytosed *R. oryzae* damages endothelial cells, and prevention of endocytosis abrogates the ability of the organisms to cause endothelial cell damage¹⁶.

Antifungal immunity requires strict regulation among different cellular processes to eliminate the fungus and protect the organism from collateral damages. In that regard, specialized proteins known as cytokines and chemokines promote a balanced communication among several cellular types, modulate phagocyte recruitment, antigen presentation, activation, and immune cell maturation as well as activation and resolution of the inflammatory response, which includes clearance of the pathogen and tissue repair. Inflammation of the lungs or bronchi brought on by the invasion of fungus into the body is the primary cause of pulmonary fungal infection. In this investigation, the effects of the fungi: *Aspergillus niger*, *A. flavus*, *Rhizopus oryzae*, and *Rhizopus delemar* on mice will be examined.

MATERIAL AND METHODS

Aspergillus niger, *A. flavus*, *Rhizopus oryzae*, and *Rhizopus delemar* isolates were gotten from smoked fish and characterized. Conidia suspension was prepared, then the number of conidia was calculated under high dry power 40X of compound light microscope, using the Eq. [1]:

$$\text{Concentration of conidia} = Z \times 4 \times 10^6 / n \quad [1]$$

where Z = total number of conidia and n = total number of small squares.

Animals

Twenty-five male white Swiss mice (*Mus musculus*) ages ranged from (8-10) weeks, (20-28) g weight, divided into five equal groups repre-

sented as: Group 1 (Control), Group 2 (*Aspergillus niger*), Group 3 (*A. flavus*), Group 4 (*Rhizopus oryzae*) and Group 5 (*R. delemar*).

The animals were fed with suitable quantity of water and complete diet. In this study, mice were intranasal exposed to *Aspergillus niger*, *A. flavus*, *Rhizopus oryzae*, and *Rhizopus delemar* conidia (25) μ L (107 conidia/mL normal saline) using micropipette every other day for 28 days which represent the test group, while control group was administrated normal saline only. After 28 days of administration, tissues were collected for biochemical analysis.

Surgical preparation and blood collection

After the exposure period, animals were anesthetized with an intra-peritoneal injection of pentobarbital and exsanguinated by cardiac puncture. Blood was collected to get serum.

Biochemical analysis

Collected blood of the mice will be centrifuged at 3000 rpm for 10 min to get the supernatant.

Oxidative stress biomarkers analyses

Serum sample was used to evaluate the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), Malondialdehyde (MDA), catalase (CAT) and the concentrations of glutathione (GSH) using the analysis kits as described by the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All analysis was performed with Mobi Microplate spectrophotometer.

Superoxide dismutase assay (SOD)

Introduction and principles

Superoxide dismutase (SOD), usually catalyses the dismutation of the superoxide anion ($O_2^{\cdot-}$) into H_2O_2 and O_2 , consequently, SOD is one of the highly recommended antioxidant available. SOD can inhibit the autoxidation of pyrogallol in the presence of ethylenediaminetetraacetic acid (EDTA) at pH 8.2. The principle of the technique mainly rely on the competition between the pyrogallol autoxidation by $O_2^{\cdot-}$ and the dismutation of this radical by SOD^{17,18}.

Materials

SOD Assay buffer, SOD Chromogen powder (Pyrogallol), and SOD Chromogen diluent (HCL solution)

SOD Chromogen solution preparation and sample pre-treatment

Serum was used directly without any

pre-treatment. Reconstitute the SOD Chromogen Powder by combining SOD chromogen powder and its diluent.

Assay procedure

Clean cuvette was filled with about 50 μL of the analyte, followed by addition of 1 mL of SOD assay buffer. About 1 mL of SOD chro-

mogen solution was then added to the cuvette before thorough agitation, absorbance was recorded at 420nm immediately, then read again at one-minute interval. For blank measurement, sample was replaced with 50 μL distilled water and follow steps 2-4.

Calculation

Change in absorbance of samples and blank was determine by using Eqs. [2-5]:

$$\begin{aligned} \text{Change in absorbance of sample } (\Delta S) = \\ \text{Absorbance of sample (1 minute)} - \text{Absorbance of sample (Initial)} \quad [2] \end{aligned}$$

$$\begin{aligned} \text{Change in absorbance of blank } (\Delta C) = \\ \text{Absorbance of blank (1minute)} - \text{Absorbance of blank (Initial)} \quad [3] \end{aligned}$$

$$\text{Percentage inhibition of pyrogallol autoxidation} = (\Delta S / \Delta C) \times 100 \% \quad [4]$$

$$\text{SOD activity (U/mL)} = \% \text{ inhibition of pyrogallol autoxidation} / 50\% \quad [5]$$

All analysis was performed with Molecular Devices SpectraMax Plus 384 Microplate Reader (Sunnyvale, CA).

Catalase assay (CAT)

Introduction

Catalase is an enzyme that breaks down H_2O_2 into H_2O and O_2 . As a by-product of numerous oxidase and superoxide dismutase processes in the eukaryotic cell, hydrogen peroxide is generated. The build-up of hydrogen peroxide promotes oxidation of biological targets such as lipid, DNA and proteins resulting in cell death and mutagenesis. Catalase protects the cell by removing H_2O_2 .

Principles

This test procedure relies mainly on measuring the amount of H_2O_2 substrate left after catalase activity. Catalase transforms hydrogen peroxide to water and oxygen (catalytic route), and subsequently sodium azide stops the enzymatic

activity. A colorimetric approach was used to test an aliquot of the reaction mix for the presence of hydrogen peroxide. In the presence of hydrogen peroxide and horseradish peroxidase (HRP), a substituted phenol (3,5-dichloro-2-hydroxybenzenesulfonic acid) links oxidatively to 4-aminoantipyrine to create a red quinoneimine dye (N-(4-antipyrinyl)-3-chloro-5-sulfonatep-benzoquinone-monoimine) that absorbs at 520 nm¹⁹.

Materials

Catalase assay buffer, catalase chromogen, stop solution, hydrogen peroxide solution for calibration curve, and substrate solution

Standard curve preparation

Calibration curve was prepared by diluting the calibration curve substrate (10 mM H_2O_2) as in the Table 1.

Volume of 10 mM H_2O_2 (μL)	Buffer (μL)	Standard Solution containing H_2O_2 (mM)	Amount of H_2O_2 in the Mixture (mM)
0	1000	0	0
125	875	1.25	0.0125
250	750	2.5	0.0250
500	500	5.0	0.0500
750	250	7.5	0.0750

Table 1. Standard curve for catalase assay.

Varying concentrations of H_2O_2 standard solutions was prepared by preparing 0, 125, 250, 500, and 750 μL of 10 mM H_2O_2 solution in reaction tubes before adding assay buffer to a final volume of 1.0 mL before agitation by inversion. Ten μL of aliquot of each solution was transferred to another reaction tube before adding 1 mL of the colour reagent. Absorbance at 520nm was taken after 15 min waiting period. Different standard solutions of H_2O_2 were prepared fresh every day. Standard curve plot of the absorbance taken at 520 nm against the final volume of H_2O_2 in the reaction mixture was made.

Assay procedure

The assay procedure was carried out at approximately twenty-five degree Celsius ($\sim 25^\circ\text{C}$). Colorimetric Assay Substrate Solution, the Assay Buffer and Colour Reagent were allowed to equilibrate to room temperature.

Appropriate volume (25 μL) of sample was added to a test tube. This was followed by add-

ing 75 μL of assay buffer to the test tube. The reaction was then started by addition of 25 μL of the Colorimetric Assay Substrate Solution to the test tube. The mixture was inverted and incubate for 15 min for proper mixing. The reaction was stopped by adding and mixing 825 μL of the stop solution to the mixture. Ten μL of the aliquot of the reaction mixture was added to different test tube before adding 1 mL of the Chromogen Reagent. It was agitated by mixing vigorously. This was followed by at least 15 min wait at room temperature for colour development. One mL of the resulting solution was transferred and then followed by absorbance measurement at 520 nm.

Calculations

Quantity of H_2O_2 (mmoles) left in the colorimetric reaction mixture was determined with the H_2O_2 standard curve. Value calculated from the above was used to determine the catalase activity (Eq. [6]):

$$\text{Activity} = (\Delta \mu \text{ moles } \text{H}_2\text{O}_2 \times d \times 100 / V \times t) \mu \text{ moles/min/mL} \quad [6]$$

Where $\Delta \mu \text{ moles } (\text{H}_2\text{O}_2)$ = change in volume of H_2O_2 added to the colorimetric reaction between the blank and a given sample, d = addition of original sample for catalase reaction; t = catalase reaction duration for catalase which was 15 min; V = volume of the sample in catalase reaction (0.025 mL); 100 = dilution of aliquot from Catalase Reaction (10 μL from 1 mL)

At a substrate concentration of 50 mM hydrogen peroxide, one unit of catalase will degrade 1.0 micromole of hydrogen peroxide to oxygen and water every minute at pH 7.0 at 25°C . All analysis was performed with Molecular Devices SpectraMax Plus 384 Microplate Reader (Sunnyvale, CA).

Glutathione assay (GSH)

Glutathione assay was done using serum sample. Method to analyse the reduced glutathione (GSH) level as described earlier by Ellman²⁰ and Alisik *et al.*²¹ was used. Thiols react with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB), breaking the disulfide interaction that produce 2-nitro-5-thiobenzoate (TNB^-), which ionizes to the TNB^{2-} dianion in water at both alkaline and neutral pH.

Fifteen μL of hemolysates were combined with 260 μL of assay buffer (0.1 M sodium phosphate and 1 mM EDTA, pH: 8) and 5 μL

of Ellman reagents to determine GSH levels in the samples. TNB^{2-} formation was measured in a spectrophotometer by measuring the absorbance of visible light at 412 nm after samples were incubated for fifteen min at room temperature. The absorbance readings were compared to a standard curve produced from a recognized GSH standard curve. All analysis was performed with Molecular Devices SpectraMax Plus 384 Microplate Reader produced in Sunnyvale, CA.

Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) level was evaluated as reported by Flohé and Günzler (1984)²² using the Glutathione Peroxidase Assay kit (Catalog No. 703102, Cayman Chemical, Ann Arbor, MI, USA). Reduced glutathione (GSH) is oxidized to glutathione disulfide (GSSG), whereas NADPH is converted to NADP^+ by GPx. During the oxidation of NADPH to NADP^+ , the absorbance at 340 nm will decrease. When GPx activity is rate restricted, the sample's GPx activity is proportional to the rate of A340 drop. At 25°C , one unit of GPx is defined as the amount of enzyme required to convert 1.0 nmol of NADPH to NADP^+ per minute. All analysis was performed with Molecular Devices SpectraMax Plus 384 Microplate Reader produced in Sunnyvale, CA.

Lactic acid dehydrogenase (LDH)

The interconversion of lactate and pyruvate is catalyzed by LDH, an oxidoreductase. The reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in a NADH-coupled enzymatic reaction to a reduced form of MTT with an absorption maximum at 565 nm is the basis for this non-radioactive colorimetric LDH test³². The purple colour intensity formed as a result of the reaction is a direct correlate of the enzyme activity. All analysis was performed with Molecular Devices SpectraMax Plus 384 Microplate Reader (Sunnyvale, CA).

Malondialdehyde (MDA) analysis

Malondialdehyde (MDA) is a naturally occurring lipid peroxidation end product that may be measured via a controlled reaction with thiobarbituric acid to produce Thiobarbituric acid reactive species (TBARS). TBARS measurement is a screening and monitoring approach for lipid peroxidation that involves measuring the absorbance of TBARS concentration at 530–540 nm as reported by Janero³⁴. Experimental variable was reported as mole equivalent MDA $\mu\text{M/g}$ protein. All analysis was performed with Molecular Devices SpectraMax Plus 384 Microplate Reader (Sunnyvale, CA).

Total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) in the serum was determined and quantified as described by earlier studies²³⁻²⁵. Briefly, the experimental reaction contains 0.5 mL of a (10 mmol/L) Na-benzoate, 0.2 mL of H_2O_2 (10 mmol/L), 0.49 mL of phosphate buffer (100 mmol/L, pH = 7.4) (constituted by adding 19.5 mL of KH_2PO_4 (100 mmol/L) with 80.5 mL of Na_2HPO_4 (100 mmol/L), before the pH was adjusted to 7.4 and 0.2 mL of Fe-EDTA complex (2 mmol/L) (freshly prepared by adding same volumes of EDTA (2 mmol/L), and ammonium ferrous sulfate (2 mmol/L). Finally, the reaction mixture was left at 25 °C for 1 h.

Ten 10 μL of the test biofluid (serum) was added to the freshly prepared reactive mixture and subsequently incubated at 37 °C for 1 h. This was followed by addition of 1 mL glacial acetic acid (20 mmol/L) and 1 mL thiobarbituric acid (0.8% w/v in 100 mL of 50 mmol/L NaOH) was added, and the absorbance at 532 nm against deionised water was taken spectrophotometrically

after incubation at 100 °C for ten min in a boiling water bath after which it was cooled in an ice bath.

Each sample (labelled as A1) had its separate control (labelled as A0, sample blank), in which the Fe-EDTA combination and H_2O_2 was added after the twenty percent acetic acid has been applied. A negative control (labelled as K1 and K0) was generated (for at least in triplicate) for each series of analysis, using the identical reagents as A1 or A0 except phosphate buffer instead of serum. For calibration, 1mmol/L uric acid standards (UA1 and UA0) were utilized.

TAC was computed with the formula stated below (Eq. [7]):

$$\text{TAC (mmol/L)} = \text{CUA (K - A)/K - UA [7]}$$

Where CUA (mmol/L) = concentration of uric acid, K = control absorbance (labelled as K1 - K0), A = sample absorbance (labelled as A1 - A0), and UA = uric acid solution absorbance (labelled as UA1 - UA0)

Biochemical tests were carried out using sera sample contained in a non EDTA sample bottles. Liver and kidney function tests, which include alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Gamma-glutamyl transferase (GGT), creatinine and bilirubin tests were carried out using fortress experimental kits and instructions were followed as described by the manufacturers. All analysis was performed with Mobi microplate spectrophotometer (Sunnyvale, CA).

Using a standard kit, the activity of alkaline phosphatase (ALP) was measured (Teco Diagnostics, USA). In a phosphate buffer, testicular tissue was briefly homogenized. ALP substrate 0.5 mL was poured into test tubes with labels for each sample, and 37 °C was equilibrated for 3 min. Testicular homogenates, controls, and standards totaling 50 l each were introduced to the appropriate test tube at specified intervals. For the reagent blank, deionized water was utilized as the sample. The mixture was gently stirred and incubated at 37 °C for 10 min. After that, 2.5 mL of ALP color developer was added and well mixed at specified intervals. The spectrophotometer's wavelength was adjusted to 590 nm zero with reagent blank (wavelength range: 580-630nm). Each sample's absorbance was measured and noted.

Using a standard kit, the activity of gamma glutamyl transferase (GGT) was assessed

(Pointe Scientific Inc., USA). The reagents were produced in accordance with the manual's directions. Pipetting 1 ml of the reaction mixture into tubes with the proper labels—"control" and "sample"—and pre-incubating the mixture for 5 min at 37 °C. Water was used to zero the spectrophotometer at 405 nm.

The homogenate was added and blended with 100 l. The absorbance was measured and recorded every minute for two min starting after 60 seconds. In order to get findings in U/L, the mean absorbance difference per minute (Abs./min.) was calculated and multiplied by 1158.

Heat shock protein 70

Heat shock protein 70 (HSP70) in the serum of mice were analysed with commercial murine Enzyme-linked immunosorbent assay (ELISA) kits (Adlitteram Diagnostic Laboratories, San Diego, CA, USA) as described by Mackei *et al.*²⁶. All ELISA were performed with Mobi Microplate spectrophotometer.

Enzyme-linked immunosorbent assay (ELISA) for Inflammatory Cytokines

Assessment of TNF- α

ELISA kit was used to measure the level

of TNF- (Elabsience Biotechnology Co., Ltd, USA). Each well received 100 L of the standard and sample, which were then incubated for 90 min at 37 °C. Then the liquid was taken out. After adding 100 L of biotinylated detection ab solution, the mixture was incubated at 37 °C for 1 h. Three washings were done after aspirating the fluid. The HRP Conjugate was then added, and the mixture was incubated at 37 °C for 30 min. Each well's solution was aspirated, then was washed five times. The substrate reagent was added, and the mixture was incubated at 37 °C for 15 min. The stop solution was increased by 50 L. Each well's optical density was instantly read at 450 nm, and results were then computed.

Inflammatory cytokines

Using a commercial ELISA kit, the levels of IL-1, IL-6, and NF-B in renal tissues were determined on a 96-well microtiter plate (MEIM- IAN Biological Co Ltd, China). On a microplate reader, the optical densities were read at 450 nm. From a standard curve, the concentrations (pg/ mL) were determined.

Statistical analysis

DData obtained was analysed using analysis

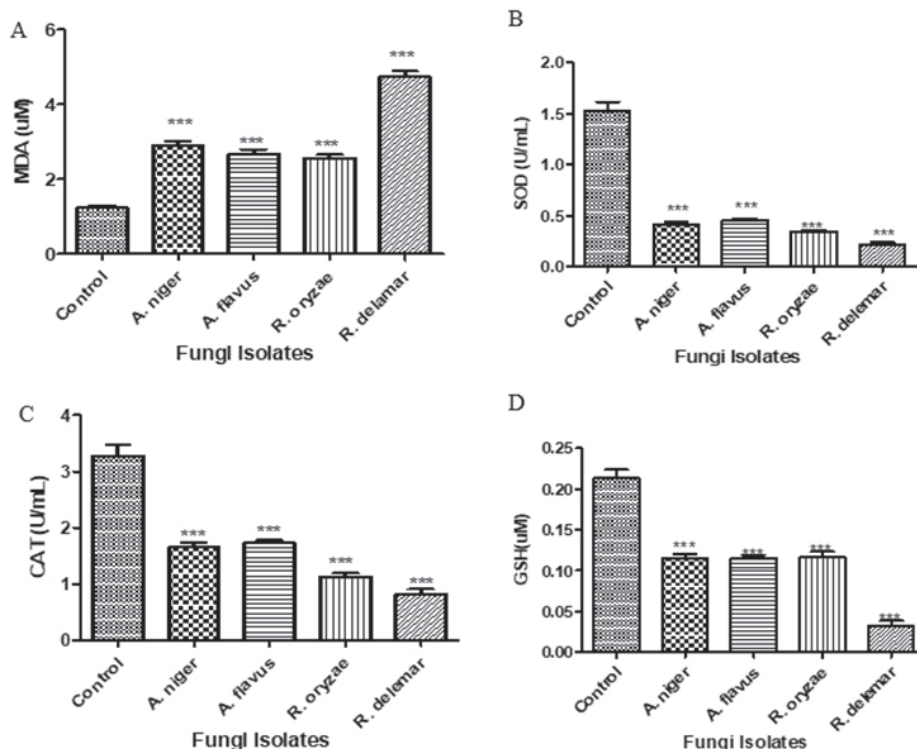


Figure 1. Enzymes activities in mice treated with different fungi isolates (mean $\pm$ SEM). a. MDA; b. SOD; c. CAT; d. GSH. *** Significant ($P < 0.001$) differences between species. Abbreviations: Malondialdehyde: MDA; SOD: Superoxide dismutase; CAT: Catalase; GSH: Glutathione.

of variance (ANOVA) and means was separated using Duncan Multiple Range Test (DMTR) with the level of significance at $p < 0.05$ (95% confidence interval).

RESULTS

Antioxidant enzymes activities

Superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) had the highest enzyme activity under control measuring 1.5U/mL, 30.2U/mL and 0.22 μ M respectively. *R. deleamar* recorded the highest level of enzyme activity for malondialdehyde at 4.3 μ M (Fig. 1A).

Total antioxidant capacity

The highest level of total antioxidant capacity (TAC) was recorded under control measuring 0.88mM/g. *A. niger* and *A. flavus* had similar TAC values at 0.37 mM/g. *R. oryzae* and *R. deleamar* recorded 0.39 mM/g and 1.9 mM/g, respectively (Fig 2.).

Lactic acid dehydrogenase activity

R. deleamar recorded the highest activity of LDH measuring 120U/L, the lowest LDH value was recorded under control at 30U/L. *A. niger*, *A. flavus* and *R. oryzae* recorded slightly different level of LDH (Fig. 3.).

Hsp70 proteins activity

The highest level of Hsp70 proteins was recorded 0.62 ng/mL under *A. niger* followed by *A. flavus* at 0.57 ng/mL. *R. deleamar*, *R. oryzae* and control recorded 0.39 ng/mL, 0.28ng/mL, and 0.17 ng/mL, respectively (Fig. 4).

Liver function test

R. deleamar recorded the highest values under AST and Creatinine (Figs. 5A & E). The liver function test had the lowest values under control for the different enzymes considered (Figs. 5A - E).

Immune responses

R. delema expressed the highest level of immune responses under IL-6, TGF-B1 and IgM. The immune responses under IL-10 and NF-KB were expressed at minimal. Immune response under INF-Y (ng/L) were expressed with slight differences for the various fungi isolates (Fig. 6).

DISCUSSION

Increase in malondialdehyde (MDA) content and antioxidant enzymes activity is an important indicator of oxidative stress. MDA is the

product formed during lipid peroxidation (LP) which cause DNA damage and cell death (Ayalá *et al.*, 2014) ²⁷. The MDA content and activities of antioxidant enzymes were determined at different times, in order to speculate the fungi isolates induced oxidative stress and effect on antioxidant defense. Results showed significant increase in MDA level in response to fungal iso-

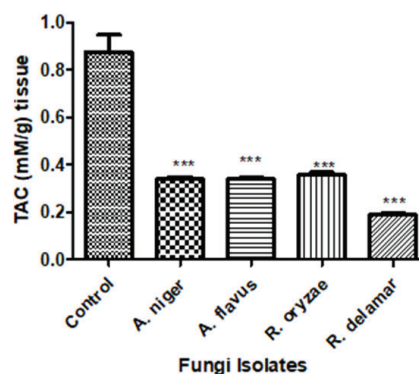


Figure 2. Total antioxidant enzymes (TAC) in mice treated with different fungi isolates mean $\pm$ SEM). *** significant ($p < 0.001$).

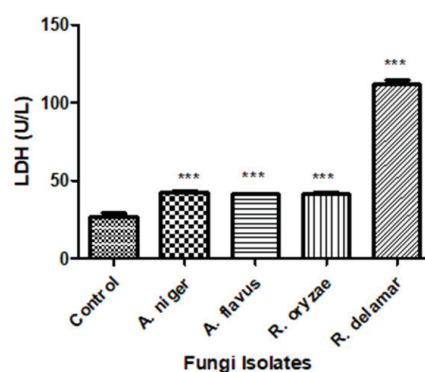


Figure 3. Activity of LDH in mice treated with different fungi isolates. *** significant ($p < 0.001$).

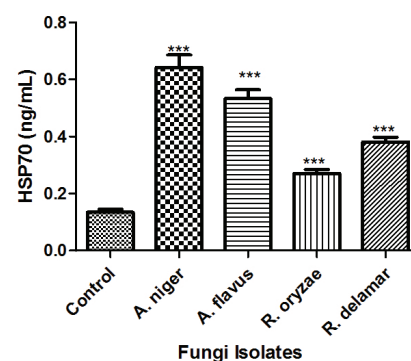


Figure 4. Activities of Hsp70 proteins, the central components of the cellular network of molecular chaperones and folding catalysts, in mice treated with different fungi isolates.

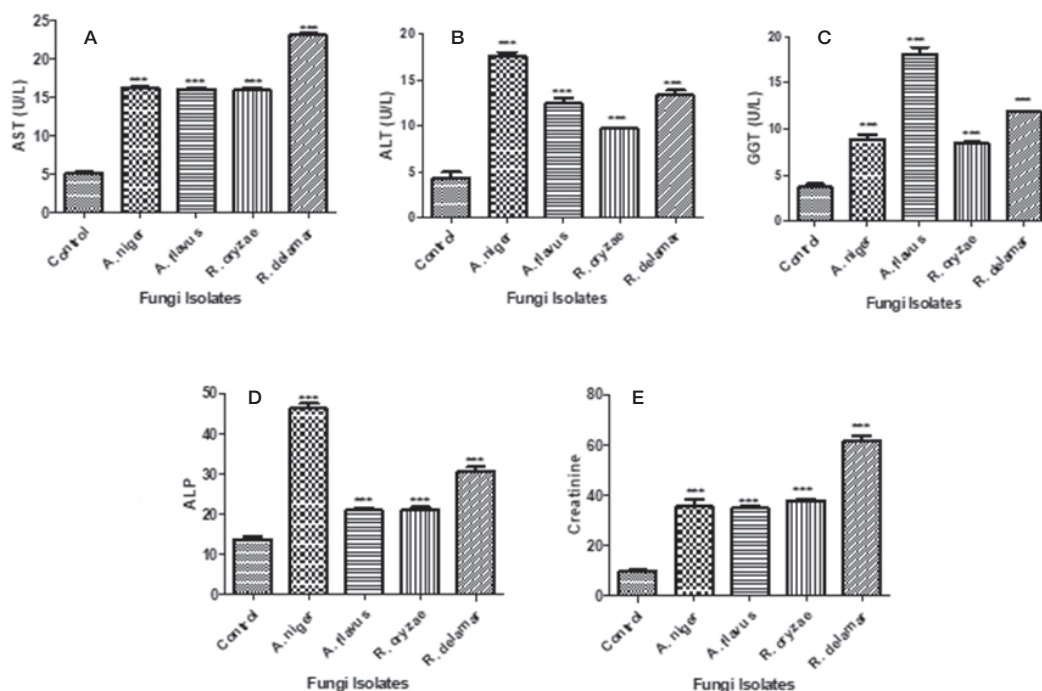


Figure 5. Liver function test in mice treated with different fungi isolates (mean $\pm$ SEM). **A.** AST; **B.** ALT; **C.** GGT; **D.** ALP; **E.** Creatine *** Significant ($p < 0.001$) differences between species. AST: Aspartate transaminase; ALT: Alanine transaminase GGT: Gamma-glutamyltransferase; ALP: Alkaline phosphatase.

lates as compared to control. Superoxide dismutase (SOD) catalyzes the conversion of two superoxide anions into a molecule of hydrogen peroxide (H_2O_2) and oxygen (O_2). In the present study, malondialdehyde (MDA) concentration in the lung tissue increased significantly. *Rhizopus delemanii* had the highest effect on MDA.

It is generally accepted that antioxidants are important reactive oxygen species counterbalance and defend the organism from exceeding oxidative stress. Reactive Oxygen Species (ROS) have been shown to be a building block of the killing response of immune cells to microbial disruption. Current affirmation has shown that ROS play a key role as an agent in normal cell signal transduction and cell cycling. Hancock *et al.*, 2001³³, in their work demonstrated that ROS have a role in cell signaling, including; apoptosis; gene expression; and the activation of cell signaling cascades. The production of SOD showed its counter effect on the activities of the reactive oxygen species. GSH abundance was more in control inoculated mice and lowest abundance was measured in *R. delemanii* inoculated tissue.

TNF is markedly induced when cells of the monocyte/macrophage lineage are coincubated with *Aspergillus* antigens or fungal elements and is markedly induced in the lungs of both

immunocompetent and immunocompromised mice after intrapulmonary challenge with conidia (Mehrad *et al.*, 1999)²⁸. The importance of TNF in the defense against *Aspergillus* in mice has since been supported by documentation that otherwise immunocompetent patients treated with TNF antagonists are susceptible to invasive aspergillosis (Rychly and DiPiro, 2005)²⁹.

Heat shock proteins (Hsps) are a family of ubiquitously expressed stress proteins and extrinsic chaperones that are required for viability and cell growth in all living organisms. These proteins are highly conserved and produced in all cellular organisms when exposed to stress. Recent reports have suggested that enhancing *in vivo* Hsp levels via non-lethal heat shock, exogenous Hsps, or plant-based compounds, could be a promising strategy used to develop protective immunity in mice against cellular stress. Iryani *et al.*, 2017³⁰ used RNAi to verify the role of HSP70 in protecting the nauplii of *A. franciscana* against abiotic and biotic stressors. The survival of nauplii lacking HSP70, compared with that of those with a functional HSP70, was decreased by 41% during heat stress and 34% upon *V. campbellii* infection. These results suggest that Hsps plays an important role in maintaining protein homeostasis by functioning as a molecular chap-

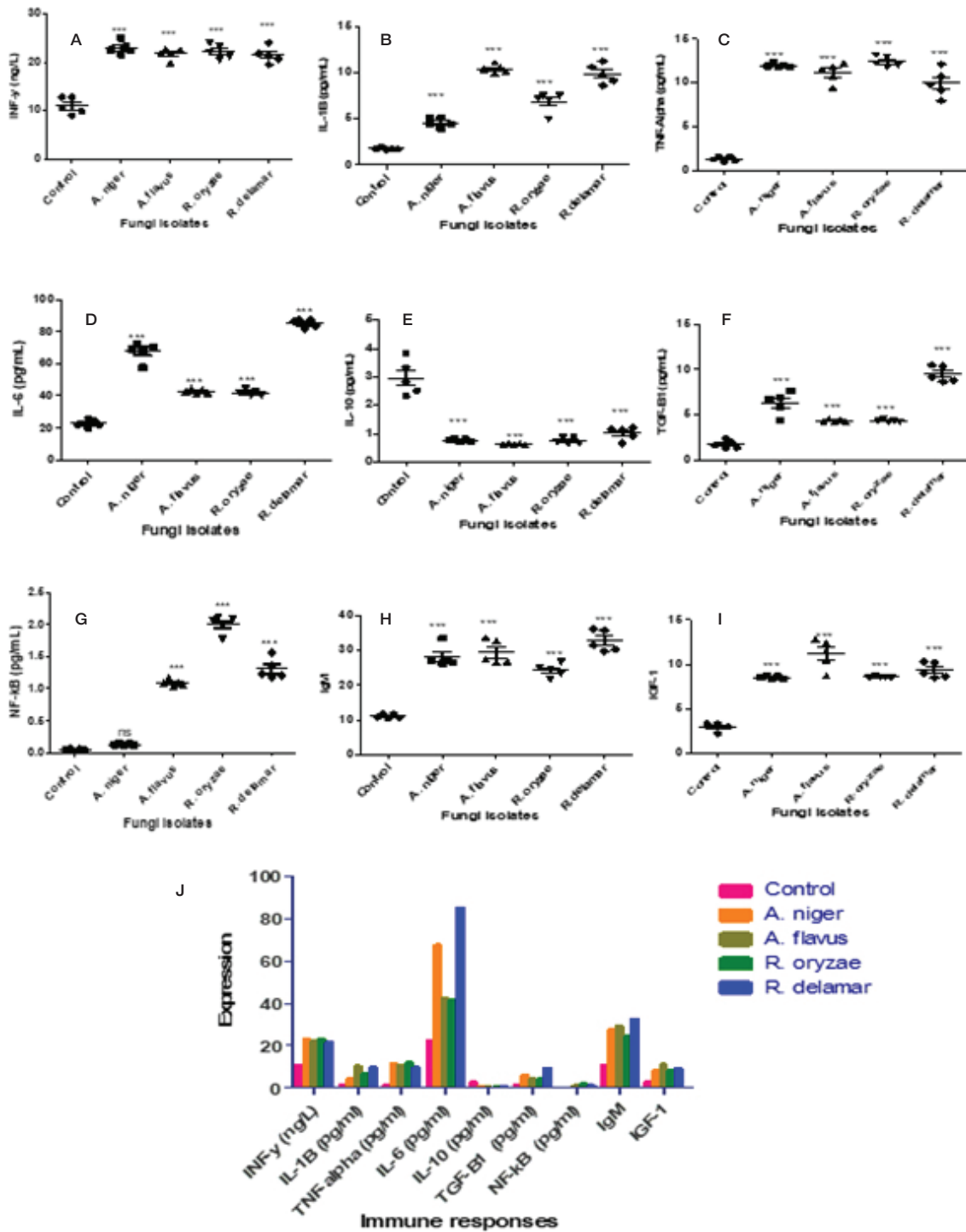


Figure 6. Different immune responses in mice treated with various fungi isolates. *** significant ($p < 0.001$) differences between species; ns: not significant.

erone, while enhancing the host innate immune response against fungal infection (Janewanthanakul *et al.*, 2019)³¹. Results of HSP70 in this study also supports these reports as high level of protein activities were observed for the *Aspergillus sp.* Additionally, Hsp70 is also reported to

induce thermotolerance, protect against osmotic stress, prevent oxidative toxicity and damage, and improve tolerance against microbial infection. These observations clearly illustrated that Hsps play a significant role in host immunity and health.

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